

BIOCHEMICAL AND HISTOCHEMICAL STUDIES
OF THE PHOTORECEPTOR CELLS
AND THE INTERPHOTORECEPTOR MATRIX
OF THE BOVINE RETINA

by

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degree of PhD
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1990.



Declaration

I certify that I am the author of this thesis and that
all the work included is my own unless otherwise
indicated in the text.

To M & P

Acknowledgements

I should like to thank Dr Jeff Haywood for all his help, advice and discussion throughout the supervision.

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Thanks to Dr R. Longshaw and Dr I. Gilmore for all their technical and Professor JV Forrester for the use of his electron microscope and image

analysers. Thanks is also given to Dr H. Kelly for synthesising and purifying the synthetic peptide.

Finally I thank the British Retinitis Pigmentosa Society for funding this project.

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ABSTRACT

Much interest has been shown recently in the components of the surfaces of the rod and cone photoreceptor cells in the mammalian retina and in the constituents of the interphotoreceptor matrix (IPM) which interact with the surfaces of the photoreceptor cells. Such molecules are likely to assist in maintaining the structures of the photoreceptor cells and/or to have functional roles in the continual interactions between the pigmented epithelium cell layer and the neural retina.

Abnormalities in the composition of such molecules may lead to disease states in which such structures and functions have broken down.

One approach to identifying constituents of the surfaces of cells is to use cell-specific probes in the form of monoclonal antibodies which can be raised against cell-specific surface molecules, and may be used to identify these molecules and to allow comparisons to be made between normal and diseased tissue.

Mice immunised with partially purified surface molecules of photoreceptor outer segments or with a synthetic peptide corresponding to the N-terminus of a cone-specific rhodopsin-like protein, gave rise to the production of monoclonal antibodies with only general immunoreactivity against the retina.

hyaluronidase (Bircanoglu) had no effect on the ability of 1B81.A3 to bind, but the structure of the

Immunisation with interphotoreceptor matrix or crude photoreceptor outer segment preparations resulted in the production of hybridomas which secreted monoclonal antibodies 1001.A1 and 1001.A3.

1001.A1 binds to the interstitial retinol-binding protein (IRBP), associated with the rod photoreceptor cells and the IPM, as demonstrated by immunohistochemistry and by Western blotting and dot blotting of IPM and purified IRBP.

1001.A3 binds a soluble high molecular weight chondroitin sulphate proteoglycan.

Immunohistochemistry indicated the antigen to be present in the form of distinct sheath-like structures surrounding the photoreceptor cells. Gel filtration chromatography both in native conditions and in the presence of guanidinium chloride showed the antigen to have an apparent molecular mass of greater than 2000kDaltons and indicated the antigen was not a loosely associated aggregate of smaller components. Binding of 1001.A3 to fixed tissue sections of the bovine retina was completely abolished by their prior treatment with either chondroitinase ABC, chondroitinase AC, hyaluronidase (testicular) or trypsin. Prior treatment of tissue sections with either heparinase or neuraminidase had no effect on binding. Treatment of tissue sections with hyaluronidase (Streptomyces) had no effect on the ability of 1001.A3 to bind, but the structure of the

antigen was altered. The sheath-like structure surrounding the photoreceptors was broken down and immunoreactivity was seen in the same area of the IPM, adjacent to the photoreceptors but with no defined structure. In conclusion, the antigen is a chondroitin sulphate molecule which is associated with hyaluronic acid molecules and which together form a defined sheath-like structure surrounding the photoreceptor cells.

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SECTION 7: SUMMARY

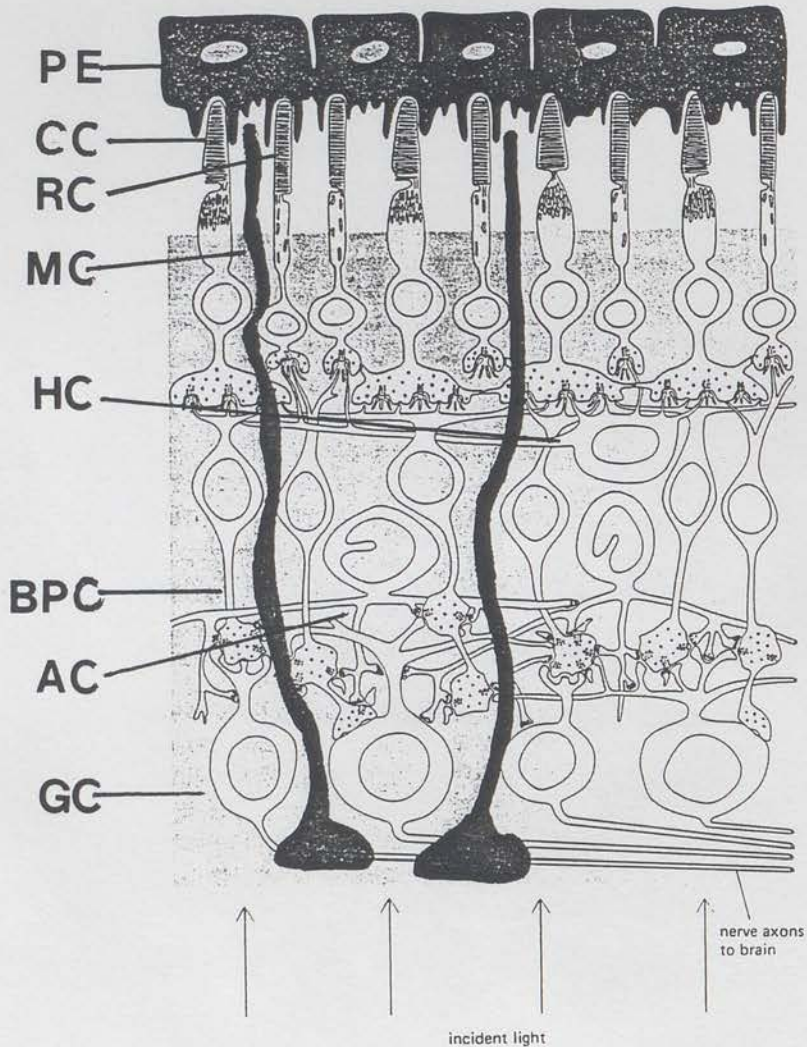
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APPENDIX.

1. Abbreviations.

1.1. References.

SECTION 1: INTRODUCTION.



PE Pigmented epithelium
 CC Cone cell
 RC Rod cell
 MC Muller cell
 HC Horizontal cell
 BPC Bipolar cell
 AC Amacrine cell
 GC Ganglion cell

Figure 1.1 Diagram of the structure of the mammalian neural retina

(Adapted from Alberts *et al* 1983)

1.1. Structure of the mammalian neural retina.

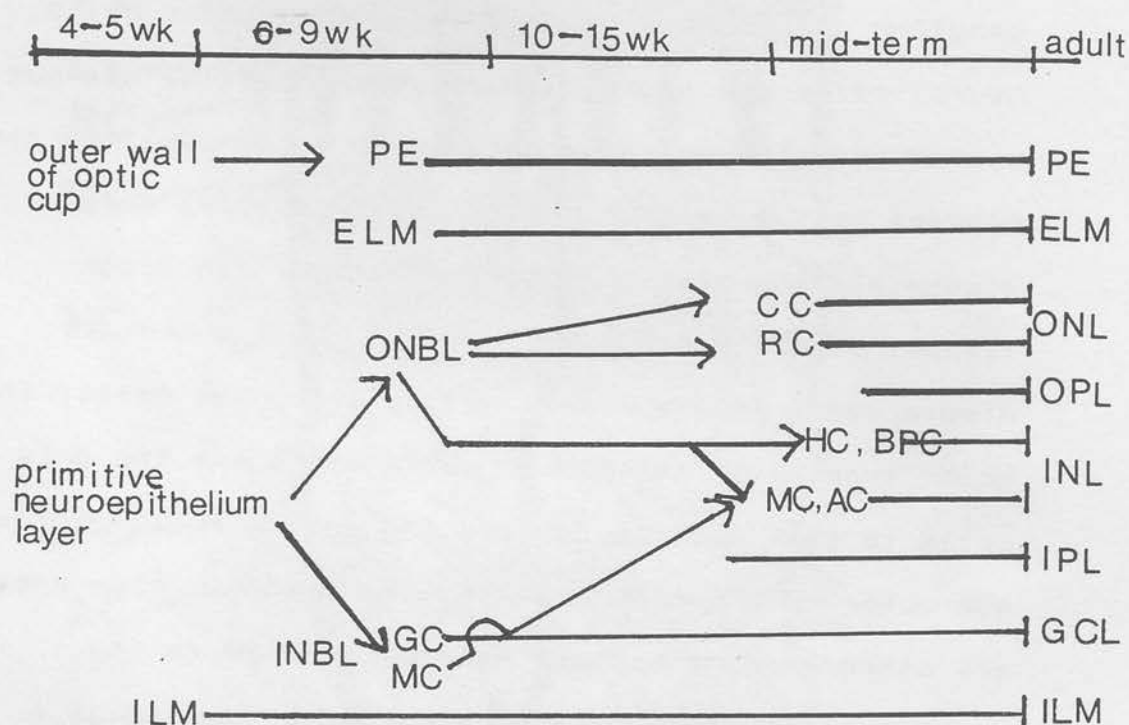
The mammalian neural retina contains five classes of neural cells: photoreceptor cells, bipolar cells, ganglion cells, horizontal cells and amacrine cells. Neural cells are found throughout the central nervous system and the retina is often used as a model for the studies of the central nervous system, being more accessible and simpler in organisation than brain tissue.

Also present in the neural retina are glial cells, the major type being the Müller cells which are the only cells to span radially across the entire retina, from the outer to the inner limiting membranes. Also present are astrocytes, which are mainly confined to the ganglion cell layer. Each neural cell type forms a layer of cells across the retina, so giving it a very ordered structure, stratified in appearance (Figure 1.1.).

1.2. Development of the neural retina.

In the vertebrate eye there are two neurally-derived tissues, the neural retina and the retinal pigmented epithelium (RPE).

During development of the human retina, the progenitors of these two tissues are initially separated. In the fourth week of gestation, these come together when the optic cup is formed. At this stage, the external, presumptive cell layer has no pigment and the retina



PE=PIGMENTED EPITHELIUM.
 ELM=EXTERNAL LIMITING MEMBRANE
 ONBL=OUTER NEUROBLASTIC LAYER
 INBL=INNER NEUROBLASTIC LAYER
 GC=GANGLION CELL
 MC=MULLER CELL
 AC=AMACRINE CELL
 HC=HORIZONTAL CELL
 BPC=BIPOLAR CELL
 RC=ROD CELL
 CC=CONE CELL
 ONL=OUTER NUCLEAR LAYER
 INL=INNER NUCLEAR LAYER
 OPL=OUTER PLEXIFORM LAYER
 IPL=INNER PLEXIFORM LAYER
 GCL=GANGLION CELL LAYER

Figure 1.2 The major steps which take place in the development of the human neural retina.

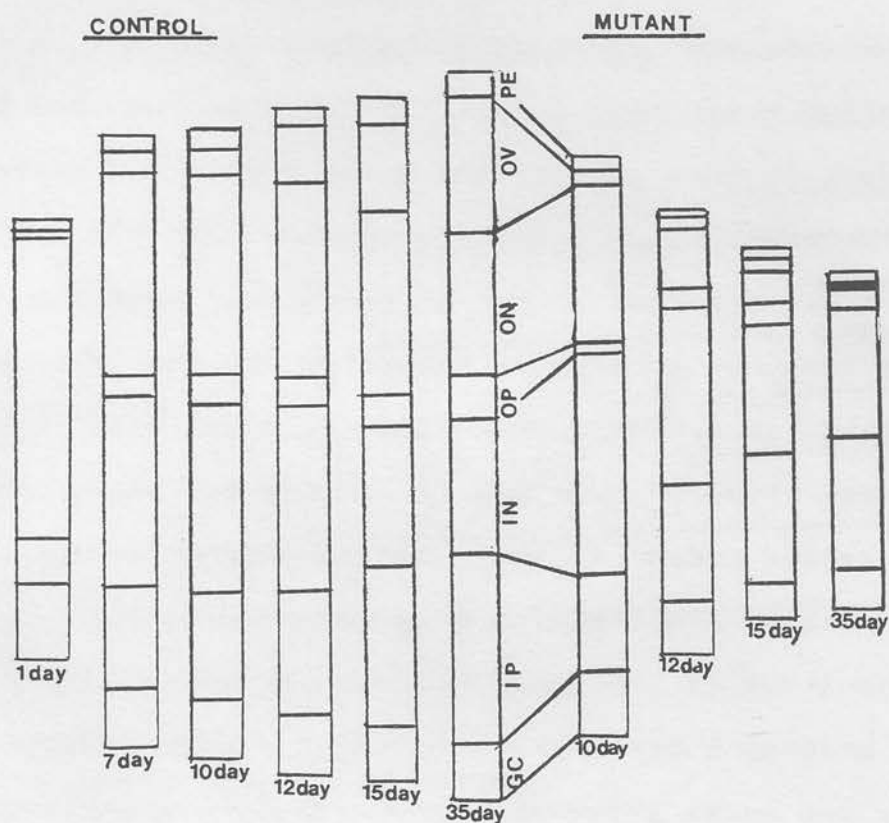
(Adapted from Rhodes 1979)

consists of an outer and an inner marginal layer. In the seventh week there is present an inner layer of mostly ganglion cells and an outer neuroblastic layer. In the ninth week of gestation there are four major horizontal layers present. There is a distinctive plexiform layer separating the inner ganglion cell layer from the outer cell layers. After this the cone cells appear, to form part of the outer row of the outer neuroblastic layer. Only by mid-term are the nuclei of rod cells apparent in the outer nuclear layer, by which time the cone axons have elongated and the pedicles widened, terminating in a widening outer plexiform layer (Rhodes 1979),(Figure 1.2.). In the following weeks, the retina widens further, the peripheral retina maturing before the macular area.

In contrast to the human retina, the mouse retina has not undergone differentiation by the time of birth. Only two weeks after birth, do the developing outer segments give the retina an almost mature appearance and development is not complete until postnatal day thirty-five (Caley et al 1974), (Figure 1.3.).

1.3. Function of the retina

The overall function of the retina is to produce chemical messages, which are understood by the brain, in response to light signals. In addition to producing a static picture of the field of vision, the retina is responsible for the perception of movement, brightness



PE Pigmented epithelium layer
 OV Outer ventricle
 ON Outer nuclear layer
 OP Outer plexiform layer
 IN Inner nuclear layer
 IP Inner plexiform layer
 GC Ganglion cell layer.

Figure 1.3 Diagram of the development of the neural retina of the normal and of the CBA rodless mouse from 1-35 days after birth.

(Adapted from Caley et al 1974)

of light and also of colour. Of the five classes of neurones present, three are directly responsible for this. Light is initially absorbed by the photoreceptor cells which synapse with the bipolar cells which in turn synapse with the ganglion cells (or in the case of the rod stimulated bipolar cells, via the amacrine II cells) (Sterling et al 1986). The final message is transported to the brain from the ganglion cells, via the optic nerve. The horizontal cells and the amacrine cells have associated regulatory functions (Kolb et al 1981, Sterling 1983). As well as enhancing stimulated bipolar cells, the horizontal cells inhibit surrounding bipolar cells so producing a greater contrast between stimulated and unstimulated cells. The horizontal cells are also regulatory and act on classes of ganglion cells including those which are direction dependent. Amacrine cells contain different neurotransmitter-like molecules such as substance P or acetylcholine. These cells terminate at one of the sub-sets of ganglion cell synapses within the inner plexiform layer (Brecha et al 1987). Cells containing one type of neurotransmitter do not contain any of the other neurotransmitter-like molecules found in the other amacrine cells (Osborne 1982). The amacrine cells are classified on this basis. The ion channels to close and the whole cell to hyperpolarise with a reduction in transmitter release (Baylor et al 1979). It is thought that an internal

1.4. Light absorbtion by the photoreceptor cells

The photoreceptor cells are responsible for the primary step of the visual cycle. It is in these cells that photons are initially absorbed and a chemical response produced (Wald 1968; Baylor 1987). The major protein of rod outer segments, opsin, binds 11-cis retinal to become rhodopsin. When light is absorbed by rhodopsin, it is bleached, that is the 11-cis retinol is converted, via several intermediates to its all-trans form (Hubbard and Kropf 1968), which dissociates from the opsin. This process leads to the catalysis of a series of reactions similar to that of the adenylate cyclase system. In the case of the rod photoreceptor cells, the photo-excited rhodopsin catalyses the release of GDP and the binding of GTP by transducin molecules. The GTP.transducin complex releases an inhibitory constraint on the cGMP-dependent phosphodiesterase and cGMP hydrolysis is initiated. The final result is a reduction in the levels of cGMP within the outer segments of light-stimulated rods.

Rods are active when unstimulated in the dark, in that ion channels in the plasma membrane of the outer segment allow a continuous influx of sodium ions, producing the "dark" or photosensitive current, and the photoreceptor cells are thereby constantly depolarised. Light causes the ion channels to close and the whole cell to hyperpolarise with a reduction in transmitter release (Baylor et al 1979). It is thought that an internal

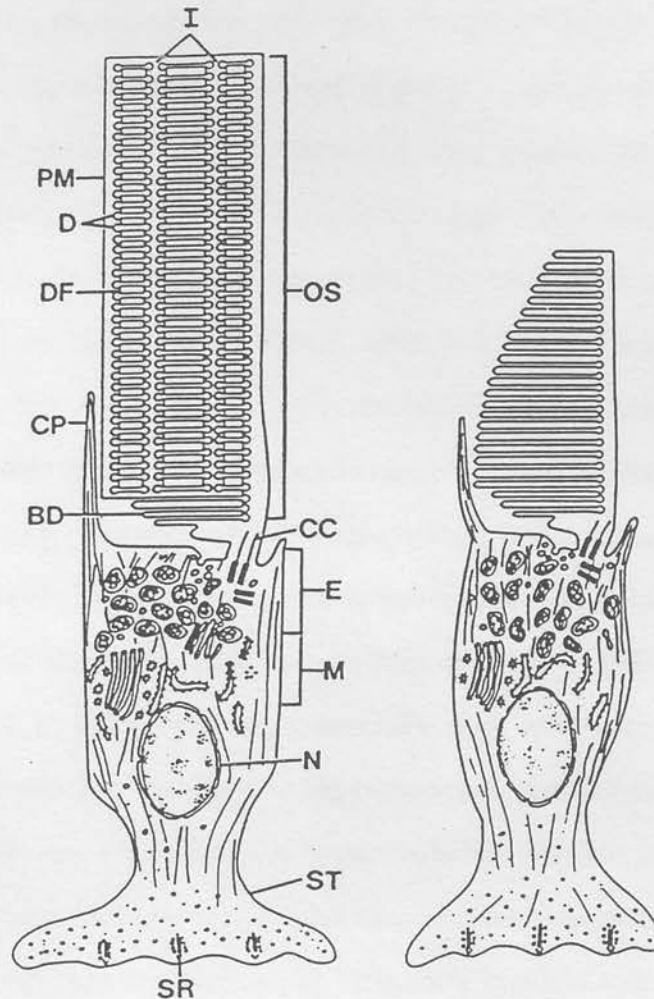


Figure 1.4. Diagram of Rod (left) and cone (right) photoreceptor cells.

The light sensitive outer segments (OS) are composed of discs (D), the main intrinsic protein being photopigment. The discs develop as evaginations of the plasma membrane of the OS base to form basal discs (BD). The rod discs are pinched off from the plasma membrane whereas the cone discs are continuous with each other. The rod discs have incisions (I) and are attached at their margins by disc filaments (DF). The OS and inner segments (IS) are linked by connecting cilium (CC). The IS contains the nucleus and is composed of an ellipsoid (E) rich in mitochondria and a myoid (M) containing organelles for the synthesis and transport of macromolecules. Calycal processes (CP) are continuous with the IS. The synaptic terminal (ST) may contain multiple synaptic ribbons (SR) which are surrounded by synaptic vesicles.

(Adapted from Bok 1985)

messenger is necessary for the transduction process and recent experiments using isolated patches of rod outer segment plasma membrane suggest that the cGMP is responsible for this, acting allosterically to close the ion channels (Fesenko et al 1985).

1.5. Structure of the photoreceptor cells

There are two types of photoreceptor cells, rods which are responsive to dim light of broad wavelength specificity, and the cones, which respond to light of different wavelengths, that is to coloured light. These two cell types are similar in structure in that both consist of an outer segment portion, an inner segment bridged by a connecting cilium and a cell body containing the nucleus. The outer segments show obvious morphological differences. They have different shapes, the rods are cylindrical whereas the cones are tapered towards their tips giving them a conical shape. The plasma membrane of the rod outer segments forms evaginations from the connecting cilium to produce internal stacks of independent plasma membrane discs along the entire length of the structure. The cones differ in that the plasma membrane of the outer segment, although evaginated along its length, is continuous and no discs are present (Figure 1.4.).

It is possible to isolate rod outer segments from the retina by loose homogenisation which releases the rod outer segment discs. These can be harvested by density

gradient centrifugation. Cone outer segments however are not found in any recognisable form. This ability to isolate rod but not cone outer segments, as well as the high average ratio of rods to cones in the mammalian retina (Marc and Sperling 1977), has meant that to date less biochemical analysis has been carried out on mammalian cones whereas the rods have been more fully characterised. Recent recombinant DNA methods have allowed some comparisons to be made between the two cell types. Genes have been cloned from each which encode similar but distinctly different proteins, for example, rod and cone cells have specific colour pigment proteins (Nathans *et al* 1986) and transducin α -sub-units (Grunwald *et al* 1986; Lerea *et al* 1986; Lerea *et al* 1989).

1.6. Interactions between the photoreceptor cells and the Retinal Pigmented Epithelium (RPE) layer

When studied by electron microscopy it is seen that the tips of the photoreceptors are in close proximity to the adjacent pigmented epithelium cell layer. These cells are continually engulfing the tips of the photoreceptors (Young and Bok 1969), which means that the photoreceptors must continually synthesise new proteins and membrane throughout life in order to maintain their own structure. When pulsed with radiolabelled amino acids, the subsequent autoradiographs showed the movement through time of newly-synthesised (radiolabelled) proteins from the inner segments towards

the tips of the rod outer segments where they were finally phagocytosed by the pigmented epithelium cells (Young and Bok 1969). In contrast, in cones, radiolabelled proteins were diffused throughout the outer segments before they were phagocytosed at the tips (Hagan et al 1974). From this it would appear that different mechanisms exist in rods and cones for the maintenance of their structure. There must be some additional (as yet unknown) mechanism in cones to maintain their tapered shape as their tips are removed. At present, the recognition signal(s) involved in the interaction between the RPE and the photoreceptors are unclear although the phagocytosis process is highly specific towards the rod outer segments (Mayerson and Hall 1986). The rhodopsin present in the rod outer segment disc membrane of the plasma membrane has been suggested as a possible ligand recognised by the RPE cells. Studies so far have shown that neither the carbohydrate groups of the rhodopsin (Shirakawa et al 1987), nor purified rod outer segment disc membranes (Laird and Molday 1988) are responsible for this. It has been suggested that some component specific to rod outer segment plasma membrane may be the ligand responsible (Laird and Molday 1988). Rod outer segment plasma membrane-specific glycoproteins have been identified (Molday and Molday 1987a; 1987b; Polans and Burton 1988; Cook et al 1989), although functions have been assigned to only some of these. The lipid

compositions of the disc membranes and the plasma membrane are also distinct (Boesze-Battaglia and Albert 1989). It has also been shown that the process of phagocytosis is under periodic control (LaVail 1976) and is independent of the visual cycle. It would seem reasonable to suggest that the surface components of the photoreceptor cells and the contents of the space between the retina and the pigmented epithelium which surrounds the photoreceptors may have some influence.

1.7. Cell surface molecules of the photoreceptors

Lectins have been used to study the cell surface molecules of the photoreceptor cells. Lectins are chemicals, usually plant derived, which bind to specific carbohydrate moieties. They therefore distinguish between glycoconjugates present on the surface of a cell which contain different carbohydrate structures.

A number of lectins have been used to study the cell surface glycoconjugates of the developing and mature retina of various species (Bridges 1981; Blanks and Johnson 1983; 1984; Uehara et al 1985;). For example, in vertebrates the lectin Concanavalin A (ConA - specific for glucose and mannose residues) binds to cells in all layers of the retina, but is most highly concentrated in the outer segments due to the large amount of rhodopsin present there (Blanks and Johnson 1983). This compares to Wheatgerm Agglutinin (WGA - specific for N-acetyl

glucosamine and sialic acid residues), which binds less strongly to the inner retinal layers whilst the outer segment reactivity is high (Blanks and Johnson 1983), again partly due to the rhodopsin which contains these sugar residues. Neuraminidase treatment reduces the outer segment binding of the lectin (Uehara et al 1985), suggesting that reactivity is due to the sialic acid component of the glycoconjugates. Wood et al state that in rat (and probably Man), trypsin digestion of the retina results in the confinement of binding to trypsin-resistant, WGA-binding domains on the distal tips of some outer segments at the outer segment/retinal pigmented epithelium interface (Wood et al 1986). These WGA-binding domains indicate the presence of a distinct population of neuraminidase-sensitive sialoglycoconjugates (Wood et al 1986).

In contrast, Peanut Agglutinin (PNA - specific for D-galactose β -1->3 N-acetyl-galactosamine-galactose residues) does not bind to rhodopsin and reacts with a sub-population of the photoreceptor outer segments, postulated to be cone outer segments (Bridges 1981; Blanks and Johnson 1983; Uehara 1983; Uehara et al 1985; Hageman and Johnson 1986). This is true for many species including fish, rabbit, monkey, human, chick, turkey, rat and dog (Blanks and Johnson 1984; Hageman and Johnson 1986). Improved embedding and fixation processes allowed light microscopy at a higher resolution to be carried out. This showed the PNA-

binding material to be present in the interphotoreceptor matrix, in the form of distinct domains which ensheath the vertebrate cone photoreceptors, rather than to be a structural part of the cones themselves (Johnson et al 1985; Johnson et al 1986). Oblique tissue sections show this PNA-binding cone extracellular matrix sheath to be composed of structural entities which are sensitive to trypsin but not to chondroitinase ABC, chondroitinase AC or heparinase (Johnson et al 1986), suggesting that glycosaminoglycans are not major sheath components. PNA also binds to cone cell body and axonal membranes, to cone synaptic pedicles located in the outer plexiform layer, to regions within the inner plexiform layer, and to the inner limiting membrane (Blanks and Johnson 1983; 1984). It has been suggested that some retinal PNA-binding glycoproteins might serve as specific molecular markers for cone photoreceptor cells.

To date much less is known about the molecular composition of the cone photoreceptors than about the rods. Differences in the chemical reactions and control mechanisms within the two cell types have yet to be studied. A 33kDalton fucosylated glycoprotein has been identified in the cone photoreceptors of fish, which is not present in the rods. The PNA-binding properties of this molecule have not been determined (Bunt and Saari 1982). Similarly, there is a preferential uptake of radiolabelled galactose in the bovine cones but not in rods (Keegan et al 1985). Again the PNA-binding

properties of the final synthesised product have not been studied and it is not known if this is the PNA-binding material associated with the cones. Many attempts have been made to isolate and characterise the PNA-binding glycoproteins present in the cone sheaths of the interphotoreceptor matrix. The literature concerning these glycoproteins is somewhat confusing. Some reports indicate the presence of high molecular weight PNA-binding glycoproteins (Uehara et al 1983; 1986b). Western blots of monkey retinal extracts were treated with PNA and identified a 160kDalton glycoprotein, although PNA-affinity chromatography failed to isolate this molecule. Polyclonal antibodies raised against this molecule recognise molecules present on both the rod and cone cells (Uehara et al 1986a). A PNA-binding glycoprotein with a molecular weight similar to that of the interstitial retinol binding protein (IRBP) has been identified in the human retina (Shuster et al 1987). This molecule has a distribution pattern distinct from that of IRBP as it is associated with both the rods and cones whereas IRBP is associated with the rods only. The fact that PNA binding is limited to the cones and that this high molecular weight PNA-binding glycoprotein is found to be associated with both the rod and cone cells may indicate that not all forms of the molecule are glycosylated to the same extent and that forms which do not bind PNA are associated with the rod photoreceptors, whilst the

other(s) is/are seen associated with the cones. Other studies have indicated the presence of predominantly lower molecular weight PNA-binding glycoproteins (Johnson et al 1985; Hageman and Johnson 1986), with varying degrees of glycosylation (Uehara et al 1986b). These are largely conserved throughout many species including turkey, rat, dog, pig, monkey and human. The fact that many of these molecules are susceptible to trypsin, and that the PNA-binding sheaths surrounding the cone photoreceptors are also susceptible to trypsin would suggest that these isolated PNA-binding glycoproteins are present within the sheath structures in vivo (Hageman and Johnson 1986).

1.8. The interphotoreceptor matrix

The space between the photoreceptor cells and the pigmented epithelium cell layer is known as the interphotoreceptor matrix (IPM) (Röhlich 1970). In contrast to the material in the extracellular matrix of connective tissues, studies have indicated that neither fibronectin nor collagen are present in the IPM (Adler and Klucznik 1982; Kohno et al 1983) and that the soluble protein fraction of bovine IPM consists of protein and glycoprotein (98% w/w) and glycosaminoglycans (2% w/w) (Adler and Klucznik 1982; Adler and Severin 1981). The IPM is critical in the transfer of nutrients and metabolites to and from the photoreceptors since these cells rely predominantly on the choroidal

capillaries behind the RPE rather than the blood vessels of the retina itself. Components of the IPM must also have structural roles with respect to the maintenance of the shapes of the photoreceptor cells. The IPM has also been implicated as having a role in retinal adhesion, phagocytosis and retinoid transfer (Zimmerman and Eastham 1959; LaVail et al 1981; Adler and Klucznik 1982; Fong et al 1984; Okajima et al 1989) although the functions of many of the individual IPM molecules are not known for certain.

There is also uncertainty as to where most of the constituents of the IPM are synthesised. It is most likely that the surrounding photoreceptor, Müller or RPE cells are responsible for their synthesis and subsequent secretion into the IPM (Adler and Severin 1981).

Initial studies of the constituents of the IPM concentrated on the glycosaminoglycans present in it. Histological studies were carried out with the use of cationic dyes to demonstrate the presence of glycosaminoglycans with dyes such as ruthenium red, alcian blue, colloidal iron and silver methanamine used to identify polysaccharide-rich molecules (Zimmerman and Eastham 1959; Röhlich 1970; Feeney 1973).

Further analysis of the glycosaminoglycans involved the chemical fractionation and characterisation of the glycosaminoglycan chains (Bach and Berman 1971a; 1971b).

The ability of the neural retina to incorporate radiolabelled sulphate and to deposit radiolabelled sulphated glycosaminoglycans into the medium indicated that cell types within that tissue were responsible for their presence in the IPM (Hall et al 1965; Occumpaugh and Young 1966; Morris 1984). Similar types of studies have also implicated the cells of the RPE as a source of glycosaminoglycans in the IPM (Zimmerman and Eastham 1959; Röhlich 1970; Stramm 1987). It is, of course, likely that both sources provide the IPM with a distinct set of glycosaminoglycan molecules.

More recent histological studies of the IPM have used the dyes cuprolinic blue (Tawara et al 1988) and cupromeronic blue (Tawara et al 1989) which may be used at a critical electrolyte concentration to stain sulphated polyanions. Such studies have indicated the presence of chondroitin sulphate-type molecules in distinct areas within the IPM. Large aggregates surrounding the photoreceptor cells were identified, as well as smaller filaments in the area of the IPM adjacent to the RPE. No staining was apparent within the photoreceptor cells whereas staining was seen within the RPE cells. It is possible that the RPE cells are responsible for the synthesis and the subsequent secretion of these chondroitin sulphate-type molecules in the form of the smaller filaments and that these

filaments then aggregate to form the large mass of chondroitin sulphate-type structures surrounding the photoreceptor cells.

Immunocytochemical analyses of the IPM have also been carried out using monoclonal antibodies raised against chondroitin sulphate molecules after digestion to remove their sulphate groups. It was found that rat IPM contains 6-sulphated chondroitin sulphate and also unsulphated chondroitin proteoglycans in distinct regions of the IPM, and does not appear to contain 4-sulphated chondroitin sulphate or dermatan sulphate proteoglycan (Porello and LaVail 1986; Porello et al 1989). In primates it was found that chondroitin 6-sulphate glycosaminoglycan is a major constituent of the cone photoreceptor matrix sheath (Hageman and Johnson 1987). From this it would appear that physical interactions may occur between certain types of glycosaminoglycan structures and specific molecules associated with the surfaces of the different photoreceptor cell types, for example the PNA-binding glycoproteins discussed in 1.7, and primate chondroitin 6-sulphate proteoglycans would appear to be co-localised, in the area surrounding the cone photoreceptor cells.

Studies of the proteins and glycoproteins within the IPM are less extensive. Proteins have been identified by SDS-polyacrylamide gel electrophoresis and many are conserved between a wide variety of species (Adler et al

1988). The functions of these molecules are largely unknown although the most abundant glycoprotein present in the IPM, the interstitial retinol binding protein (IRBP) has been well characterised. Antibodies however,

has shown IRBP to be confined predominantly to the IPM.

1.9. The interstitial retinol binding protein

The interstitial retinol binding protein (IRBP) is the major component of bovine IPM where it is present at a concentration of 30-100uM (Adler and Klucznik 1982). Its molecular weight on calibrated gel filtration columns appears to be 249 000 Daltons while SDS-polyacrylamide gel electrophoresis gives an apparent molecular weight of 144 000 Daltons suggesting that it may exist as dimers (Fong et al 1984a). Bovine IRBP contains 8.4% (w/w) carbohydrate, consisting of sialic acid, neutral hexoses and glucosamine in a molar ratio of 1:3:2 and it has at least four Concanavalin A binding forms with pI values ranging from 4.4 to 4.8 (Fong et al 1984a). An analogous protein has been isolated from human IPM with an apparent molecular weight of 135 000 Daltons.

Studies with lectins suggest that at least one of its oligosaccharide chains is a sialated biantennary complex type containing fucose (Fong et al 1984b). Monkey neural retinas incubated in vitro in media containing [3 H]Fucose (Holleyfield et al 1985), or [3 H]Leucine (Fong et al 1984b) and also bovine retina incubated with

protective role preventing the oxidation of the retina

[³H]Fucose (Fong et al 1984a), have both been shown to synthesise and secrete radiolabelled IRBP into the medium.

Immunocytochemistry using polyclonal antibodies however, has shown IRBP to be confined predominantly to the IPM (Fong et al 1984b, Schneider et al 1986). It may be that the antigenic determinants are somehow masked, or that the protein is in too low a concentration within the cells of the retina to be detected by these means. The cellular origin of IRBP was found from the distribution of a cDNA clone of the IRBP gene. This was restricted to rod outer segments and to pinealocytes (van Veen et al 1986).

Bovine IRBP binds two molecules of all-trans retinol (Fong et al 1984a) and is the only retinol-binding species in the interphotoreceptor matrix (Pfeffer et al 1983). It also binds exogenous cholesterol, α -tocopherol and all-trans retinoic acid, all of which are completely displaced by all-trans retinol (Fong et al 1984a). The amount of retinol bound to IRBP depends on the proportion of bleached rhodopsin in the retina. Light-adapted IPM contains much more bound retinol than does dark-adapted IPM (Adler and Martin 1982). It is believed that IRBP may be involved in the transfer of retinol between the RPE and the retina during the visual cycle. It has been suggested that it may also have a protective role preventing the oxidation of the retina

1983), the rodless CRA mouse (Coley et al 1976) (Figure

by retinol, which itself is a highly toxic molecule, or by other exogenous materials such as α -tocopherol (Fong et al 1984a).

In the case of the RCS rats, the disease is associated

1.10. Abnormalities of the retina

There are many genetic diseases involving abnormalities of the retina which ultimately lead to blindness.

Retinitis Pigmentosa is the collective name for many such diseases. The disease often becomes apparent in the early twenties when the degeneration of the photoreceptors is observed leading to a pigmented effect on the retina. There is a preferential loss of rod photoreceptors, the cones remaining intact for much longer periods. Degeneration is gradual and finally leads to blindness after many years of a gradual loss of sight.

The disease often becomes apparent later in life and is not a fatal condition, and so it is rare to obtain affected eyes for research purposes, especially those which are at an early stage of the disease. Much research has therefore turned to the use of suitable animal models.

The retina of rodents provides a convenient system in which to study the development of healthy and diseased retina because of its rapid postnatal development (1.2.). There are several inherited retinal defects such as the retinal degenerate mouse (Sidman and Green 1965), the rodless CBA mouse (Caley et al 1974) (Figure

1.4.), the Purkinje cell deficient (pcd) mouse (LaVail et al 1982) and the Royal College of Surgeons (RCS) rat (Bok and Hall 1971).

In the case of the RCS rats, the disease is associated with a mutation in genes of the cells of the pigmented epithelium, which ultimately leads to the degeneration of the photoreceptor cells. Here the pigmented epithelium is unable to phagocytose the rod outer segments when they are shed by the photoreceptors. As a result, photoreceptor fragments accumulate in the IPM, debris builds up and the photoreceptors begin to degenerate. Within one month, only the cone photoreceptors remain. If the IPM plays a key role in normal photoreceptor physiology it is important to study the IPM in those diseased states in which the photoreceptors degenerate. Histochemical studies using alcian blue, toluidine blue and colloidal iron to detect glycosaminoglycans, metachromatic staining substances and highly negatively charged substances respectively have been used to follow the development of the retina of the RCS rat (LaVail et al 1981; Cohen and Nir 1984). This showed the normal intense staining of the IPM next to the retinal pigmented epithelium to be incomplete in RCS rats and that this staining disappears as debris builds up. The basal outer segment staining however is greater in RCS rats presumably due to the abnormal accumulation of IPM in this region. It is possible that this IPM distribution alters the normal

diffusional processes, resulting in photoreceptor cell death. For example, proteoglycans of the IPM may form some physical barrier and may cause either the accumulation of metabolites next to the photoreceptors, or the exclusion of these metabolites from the debris. In order to understand the mechanisms of disease, a standard model of developing tissue is necessary to allow comparisons to be made between diseased and healthy tissue at specific stages of development. Cell lineage pathways have been constructed to establish the ancestry of specific cell types and to identify the progenitors of these cells (Turner and Cepko 1987; Adler and Hatlee 1989). Although these studies show where cells originate, they do not show how the differentiation process is regulated and which environmental or genetic factors have any effect.

1.11. Specific cell markers

The alternative approach to this problem is to identify cell-specific markers within the tissue. One such method is to study the carbohydrate structures of the glycoconjugates present on the surface of cells, using them to provide differentiation markers for use in the study of tissue development. Lectins have often been used for this purpose, however their uses are limited in that they do not bind specifically to single molecular structures and lectin affinity chromatography cannot distinguish between different molecules containing

similar carbohydrate moieties. For example, PNA-agarose affinity chromatography of solubilised retinal tissue would not distinguish between the PNA-binding material of the inner retina and that associated with the cone photoreceptors (1.7.). The use of further standard chemical purification steps to fractionate the mixture of PNA-binding components would lead to the isolation of a molecule of unknown source. Hence a further method of detection and identification is often required.

Two tools have recently become available for establishing this, that is, monoclonal antibodies and recombinant DNA molecules. These both offer the possibility of defining some of the molecules of importance in the retina. Both methods rely upon the ability to clone pure reagents out of heterogeneous starting mixtures. This is very important when the complexity of the tissue makes it extremely difficult to obtain any single molecule in sufficient quantity and purity for more traditional approaches. A major difference however between the two techniques is that molecules can become localised in different sub-cellular components, for example, to the outer segments of the photoreceptors or to the dendrites of nerve cells. This distinction is not picked up by DNA probes but may be by antibodies, that is, the final gene product is detected as opposed to the gene itself.

Monoclonal antibodies can be raised against specific antigens to link in vivo and in vitro studies by allowing unambiguous cell identification and studies in cellular heterogeneity, detecting gradients of expression and control of development, so leading to an understanding of these features in molecular terms. It was hoped that monoclonal antibodies would reveal unique cell surface antigens in embryogenesis, differentiation and oncogenesis. However many were found to be carbohydrate structures of glycoproteins and glycolipids occurring in many cell types (Siddiqui and Hakamori 1971; Slomiany et al 1982), suggesting important roles for the diverse carbohydrate structures as receptors involved in cell growth and differentiation. It may be that the sequential addition (or deletion) of a monosaccharide might be a mechanism for the appearance or disappearance of antigens during stages of development and differentiation (Feizi 1981; Gooi et al 1981). possibly due to differences in enzyme activities. There is the possibility that saccharides are the cell surface markers that distinguish immature from mature cells, and tumour cells from their normal counterparts. The carbohydrate sequences may have specific roles in normal cell growth and differentiation and the disruption of this may contribute to the disordered behaviour of tumour cells. Roles of the terminal galactose residues of asialo-glycoproteins as recognition structures for their clearance from serum by uptake into hepatocytes,

and the importance of phosphorylation of mannose residues on lysosomal enzymes for their correct routing to lysosomes have been well established (Ashwell and Hartford 1982).

Monoclonal antibodies would appear to be very useful tools for studies of glycoconjugates of specific cell types and to be a very sensitive method of visualising diversity in individual cells.

1.12. The use of monoclonal antibodies in studies of the neural retina

Monoclonal antibodies have been raised against the major cell classes in the retina: rod photoreceptor cells (Barnstable 1980; Fekete and Barnstable 1983; Hicks and Molday 1986; McKechnie et al 1986; Cook et al 1989); bipolar cells (Barnstable et al 1983); amacrine cells (Barnstable et al 1985); horizontal cells (Young and Dowling 1984); Müller cells (Barnstable 1980; Barnstable et al 1983; Lemmon 1985); ganglion cells (Barnstable and Dräger 1984; Fry et al 1985). Reports have been more recently made of monoclonal antibodies against the cone outer segments (Lemmon 1986; Szél et al 1986; Johnson and Hageman 1988; Lerea et al 1989).

Monoclonal antibodies have been produced against molecular markers of cell position. Such molecules have been postulated to play a role in the development of the nervous system. Antibodies have been raised against TOP molecules (Trisler et al 1986); N-CAM molecules (Noble

et al 1985); JONES molecules (Constantine-Paton et al 1986). Monoclonal antibodies have also provided developmental markers for neural retina tissue (Cole and Glaser 1984; Sarthy and Bacon 1985). Also available are monoclonal antibodies which have been raised against molecules known to be specific to a single cell type in various tissues of origin, for example intermediate filaments, which have been used to identify neural and glial cells (Hyndman and Lemmon 1975). These however have a limited use, since often the intermediate filament specific for a mature cell type appears late in development. This is true for glial fibrillar acidic protein in glial cells. In addition, monoclonal antibodies have been reported which are specific to sub-classes of a specific cell type. Young and Dowling have reported monoclonal antibodies specific to sub-classes of horizontal cells (Young and Dowling 1984). Such variation between cells presumably has some reflection on the functions of these cells.

1.13. Aims of this thesis

Little is known about the factors involved in maintaining the structure of the photoreceptors as they are continually renewed throughout life, or the factors involved in controlling the phagocytosis which occurs between the cells of the RPE and the tips of the photoreceptors. Little is known biochemically about

differences in composition between the rod and cone photoreceptors, or about the molecules in the IPM which associate with these cells. Such molecules presumably have regulatory functions with respect to the rod or cone cells.

One approach to identifying surface molecules specific to a single cell type, or to molecules associated with the cell, is to raise monoclonal antibodies.

Therefore the initial aim of this thesis was to raise mouse monoclonal antibodies against cell-specific molecules either within, or associated with, the rod and cone photoreceptor cells.

Once established, the monoclonal antibodies would be used to characterise the cell-specific molecules in biochemical terms. The distribution of the molecules would be established and further work would involve finding the distribution of the cell-specific molecules during development of the retina. This would be carried out with the view to establishing a normal pattern of development in the retina, with respect to these cell-specific molecules, before comparisons could be made with diseased retina.

Throughout the text, references are made to other sections of text both forward and backward. These are indicated by the relevant section number in parentheses; for example, (1.1.) refers to the text in Section 1.1.

2. Histological methods

2.1. Preparation of tissue sections

Bovine eyes were trimmed of excess tissue, cut in half, the vitreous humour and the anterior portion removed and the posterior portion fixed by immersion in fixative solution.

2.1.1. Fixed tissue sections

Fixation was carried out in a solution of formol calcium containing 4% paraformaldehyde (w/v), 1% calcium chloride (w/v) and 0.5% glutaraldehyde (v/v) in 0.1M sodium phosphate buffer pH7.0. The small percentage of glutaraldehyde was found necessary to maintain tissue integrity without significantly reducing its antigenicity. An overnight incubation at 4°C was sufficient to fix the tissue. The aldehyde groups of the fixative were quenched by incubating the fixed tissue in a 1M solution of sucrose containing 50mM ethanolamine pH8 and 50mM Tris HCl pH7.4, overnight. Small blocks (approximately 10mm x 3mm) were cut from the eye cup, mounted onto Tissue-Tek OCT (Miles Scientific), rapidly frozen in liquid nitrogen and stored at -20°C. Microscope slides were subbed by dipping in a 1% (w/v) gelatine solution containing 0.05% (w/v) chromic potassium sulphate dodecahydrate ($\text{KCr}(\text{SO}_4)_2 \cdot 12 \cdot \text{H}_2\text{O}$) and then air drying. 10-15 um tissue sections were cut at -10°C using a cryostat (Slee,

London), melted onto subbed slides, dried with a warm air flow and stored in a dry box at room temperature. The integrity of the retina was checked by staining with toluidine blue (2.2.4.).

2.1.2. Frozen unfixed tissue sections.

The tissue was prepared as for fixed tissue sections except that fresh blocks of tissue were immediately embedded in Tissue-Tek OCT and sections cut.

2.1.3. "Semi-thin" tissue sections

Small pieces of fixed retina were progressively dehydrated in aqueous dimethyl sulphoxide (DMSO) and embedded in Lowycryl K4M, using a variation of the method of Altman et al (Altman et al 1984). Polymerisation took place overnight at room temperature in the presence of 360nm UV light. 1 μ m tissue sections were cut using an LKB ultramicrotome.

2.2. Treatment of tissue sections

2.2.1. Antibody treatment using immunofluorescence

Each incubation was carried out at room temperature in a high humidity chamber with a 100 μ l aliquot of the incubation solution per tissue section unless otherwise stated.

Sections of the neural retina were each incubated overnight in antibody-diluting solution containing 20% (v/v) antibody-containing culture supernatant. The antibody-diluting solution consisted of TBS pH 7.4 containing 5% normal sheep serum (v/v) and 0.05% sodium azide (w/v). The antibody solution was then drained from the slide which was rinsed in TBS for 10 minutes and then drained of any excess liquid before the next incubation step took place. The second incubation was for 2-3 hours in a solution containing a 50-fold dilution of a sheep anti-mouse γ globulin-biotin conjugate (4.6.3.1.) or in a solution containing a 500-fold dilution each of commercial sheep anti-mouse IgM biotin and sheep anti-mouse IgG biotin conjugates. The slides were rinsed as above, and this was followed by a 1 hour incubation in a 50 ug/ml solution of streptavidin-FITC (4.6.2.1.). Finally the slides were rinsed, coverslipped in glycerol and viewed.

2.2.2. Immunoperoxidase staining

The first antibody incubation was carried out as described in Section 2.2.1., prior to a two hour incubation with a 50-fold dilution of sheep anti-mouse γ globulin (Scottish Antibody Production Unit, Carlukes), in antibody-diluting solution (2.2.1.). This was followed by a further two hour incubation with a 6-fold dilution of the culture medium of a mouse monoclonal antibody raised against horseradish peroxidase (HRP) (a

gift from Dr. V. van Heyningen, MRC Human Genetics Unit, Western General Hospital, Edinburgh). The final incubation was for 1 hour, with a 20 ug/ml solution of HRP diluted in TBS/1mg/ml BSA. HRP binding was visualised by the development of colour on the addition of the substrate 4-chloro-1-naphthol in a 20% (v/v) methanol solution containing 200 ug/ml 4-chloro-1-naphthol and 0.005% H₂O₂ (v/v) (final concentration) (added immediately before use). The sections were rinsed between each incubation step and coverslipped (2.2.1.) before being viewed. In experiments using sections treated by the immunoperoxidase and the immunofluorescence methods (2.2.1.), controls were carried out on sections where the first antibody was omitted from the initial incubation solution.

2.2.3. Lectin treatment

A lectin-biotin conjugate solution (4.6.3.2.) (containing approximately 1mg/ml of lectin) was diluted 100-fold in TBS containing 1mg/ml of BSA and this was incubated in 100 ul aliquots with retina sections for 1 hour prior to incubation with streptavidin-FITC, as described in Section 2.2.1. The sections were finally mounted in DPX mountant and viewed. Alternatively the sections were incubated for 1 hour in a 200-fold dilution of lectin-FITC (4.6.2.2.) in antibody diluting solution for one hour, prior to rinsing and viewing as above.

Control sections were run simultaneously where the lectin-biotin conjugate solution was omitted, or where the lectin solution was preincubated and incubated in a solution of TBS containing 1mg/ml of BSA and 200mM hapten sugar.

2.2.4. Toluidine blue staining

Sections were incubated in stain (0.25%, w/v) for 5 minutes, washed in distilled water and dehydrated in increasing concentrations of alcohol and finally cleared in xylene before being coverslipped in DPX mountant.

2.3. Treatment of "semi-thin" tissue sections

2.3.1. Antibody treatment using colloidal gold-silver enhancement

All incubations were done by placing the sections onto drops (approximately 0.2ml) of the incubation solution, placed on top of a sheet of wax proof paper. Transfer between solutions was by means of fine forceps. The sections were finally dried onto microscope slides and viewed.

Sections were treated with antibody as before (2.2.1.) except that the streptavidin-FITC conjugate solution was replaced by a 50 ug/ml solution of streptavidin conjugated to 50nm diameter colloidal gold particles. The colloidal gold treatment was followed by silver enhancement in order to visualise antibody binding. The

sections were washed for 2-3 minutes in 60mM citric acid pH7.0 and incubated in the dark in developing solution for 5-6 minutes. The developing solution was prepared in dark bottles and used immediately. 15ml of solution A (0.6M hydroquinone in 0.1M citric acid pH7.0) was added to 15ml of solution B (50mM silver lactate in citric acid pH7.0). The sections were then fixed using photographic fixing solution and finally rinsed in distilled water.

2.3.2. Lectin treatment using colloidal gold-silver enhancement.

This was carried out as for treatment with lectin (2.2.3.), except that the streptavidin-FITC solution was replaced by a solution of streptavidin-colloidal gold, which was used as described in Section 2.3.1.

3. Immunological methods.

3.1. Preparation of Monoclonal Antibodies

3.1.1. Immunisation

Ten week-old female Balb/c mice were injected subcutaneously at two sites on the flanks with a total of 100 μ l of immunogen containing approximately 100 μ g of protein, mixed with Freund's Complete Adjuvant. This was repeated four weeks later but with Freund's Incomplete Adjuvant replacing the Freund's Complete Adjuvant. After a further two weeks, the mice were injected intravenously via the tail-vein with 100 μ l of the immunogen and the cell fusion was carried out four days later.

SECTION 3: IMMUNOLOGICAL METHODS.

3.1.2. Medium

Serum-free medium consisted of RPMI1640 with 0.1% (w/v) sodium bicarbonate, 25mM HEPES and 10⁻⁶M β -mercaptoethanol. This is Dutch modification, especially suitable for lymphocytes and resistant to fluctuations in CO₂ concentrations which occur during prolonged viewing or manipulation of the cells. For selection medium consisted of serum-free medium with the addition of heat inactivated foetal calf serum to give a final 10% (v/v) concentration and also of hypoxanthine (13.8 μ g/l), aminopterin (0.17mg/l) and thymidine (3.87mg/l).

3. Immunological methods.

All media used contained the antibiotics penicillin

3.1. Preparation of Monoclonal antibodies

3.1.1. Immunisation

Ten week-old female Balb/c mice were injected subcutaneously at two sites on the flanks with a total of 100 μ l of immunogen containing approximately 100 μ g of protein, mixed with Freund's Complete Adjuvant. This was repeated four weeks later but with Freund's Incomplete Adjuvant replacing the Freund's Complete Adjuvant. After a further two weeks, the mice were injected intravenously via the tail-vein with 100 μ l of the immunogen and the cell fusion was carried out four days later.

3.1.2. Medium

Serum-free medium consisted of RPMI1640 with 0.1% (w/v) sodium bicarbonate, 20mM HEPES and 10^{-4} M β -mercapto-ethanol. This is Dutch modification, especially suitable for lymphocytes and resistant to fluctuations in CO₂ concentrations which occur during prolonged viewing or manipulation of the cells.

HAT selection medium consisted of serum-free medium with the addition of heat inactivated foetal calf serum to give a final 10% (v/v) concentration and also of hypoxanthine (13.61mg/l), aminopterin (0.176mg/l) and thymidine (3.876mg/l).

In the maintenance medium, the aminopterin was omitted. All media used contained the antibiotics penicillin (200IU/ml), and streptomycin (0.2mg/ml) and also the fungicide, Fungizone (0.25 ug/ml).

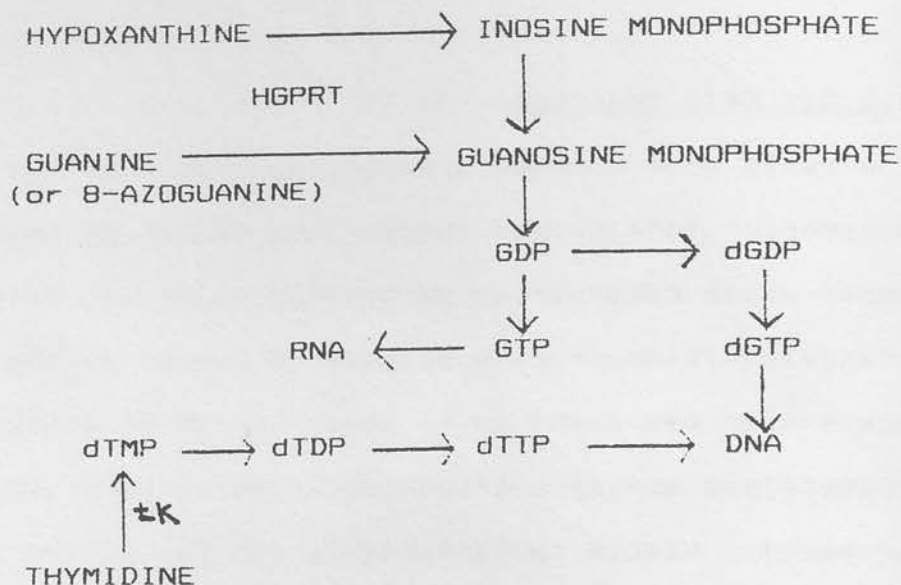
3.1.3. Cell counting

The cells were counted by applying a drop of the cell suspension to a haemocytometer and examining this either under phase contrast or by the following acridine orange/ethidium bromide method. A volume of the cell suspension was added to an equal volume of ethidium bromide and acridine orange solution (1 part per million of each). Viable cells appear green due to the accumulation of acridine orange in acidic compartments (lysosomes). Dead cells do not maintain any pH gradient and have permeable plasma membranes so allow the entry and intercalation of the ethidium bromide into the DNA and therefore appear orange (Parkes et al 1979).

3.1.4. Cell fusion

This was carried out using a variation of the method of Galfré and Milstein (Galfré and Milstein 1981).

B-Lymphocytes were obtained from the spleen of an immunised Balb/c mouse, and feeder cells (thymocytes) from the thymus of a six week-old female c57/B1 mouse. Stock myeloma cells were of the NSO line which do not secrete antibody or antibody chains and are also deficient in hypoxanthine guanine phosphoribosyl



HGPRT Hypoxanthine guanine phosphoribosyl transferase
 tk thymidine kinase

Figure 3.1 Diagram of the pathways relevant to hybrid selection in HAT medium. If the main synthetic pathways are blocked with aminopterin, cells depend on the "salvage" enzymes HGPRT and tk. HGPRT⁻ cells may be selected by growth in medium containing the toxic base analogue 8-azoguanine. HGPRT⁻ cells cannot grow in HAT medium unless they are fused with HGPRT⁺ cells.

transferase (HGPRT). To ensure no revertants (insensitive to HAT) were present prior to fusion, the NSO cells were cultured in maintenance medium (3.1.2.) containing 8-azoguanine (0.13mM), for 24 hours (Figure 3.1). This selection medium was replaced by fresh maintenance medium and the cells were grown to log phase before the fusion took place.

3.1.4.1. Day 0.

The spleen cells were teased apart by passage through a 25mm piece of nylon gauze into serum-free medium (3.1.2.). The lymphocytes were pelleted from the 10ml suspension by centrifuging at 1000g for 10 minutes. They were then resuspended in 10ml of serum-free medium (3.1.2) and the cell yield determined (3.1.3.). The NSO cells, in log growth phase, were dislodged from their flask, pelleted by centrifugation at 1000g for 10 minutes and resuspended in 5ml of serum-free medium for counting.

B-lymphocytes were fused with NSO cells at a ratio of between 10 and 20 to 1. The correct ratio of the cell types were mixed and pelleted together by centrifugation at 1000g for 10 minutes. The pellet was kept as near to 37°C as possible while 0.8ml of warm polyethyleneglycol 1540 was added dropwise over a period of 30 seconds. After a further 30 seconds of gentle mixing, 2ml of warm serum-free medium was added dropwise over a period of 2 minutes and the pelleted cells resuspended. A further

6ml of warm serum-free medium was added over 3 minutes. The cells were then pelleted by centrifugation at 1000g for 10 minutes and then gently resuspended in a suitable volume of HAT selection medium (3.1.2.) containing approximately 8×10^7 thymocytes. The thymocytes were prepared 24 hours earlier and had been incubated at 37°C in 5% CO₂). The total volume of HAT selection medium used to dilute the fused cells allowed approximately 1×10^4 potentially fused NSO cells to be dispensed in 100 ul aliquots into all the wells of five or six 96-well culture plates. Plates were incubated at 37°C in an atmosphere of humid 5% CO₂.

3.1.4.2. Day 5

Half the medium was removed from each well by aspiration and replaced by 100 ul of fresh HAT selection medium (3.1.2.).

Small colonies of growing hybridomas were usually observed around Day 8 by inverted stage microscopy.

3.1.4.3. Day 10

The cells were re-fed with maintenance medium (3.1.2.), (as by this stage all NSO cells were dead). 1-2 days after feeding, the wells were screened for anti-retinal antibody secretion (3.1.7.).

When antibody-secreting cell colonies had been identified and grown almost to confluence, they were transferred to 24-well culture plates, into a volume of 500 μ l of maintenance medium.

When the cell colonies became established in the 24-well plates, they were transferred to 25cm² flasks containing 5ml of maintenance medium (repeated testing of antibody secretion was necessary at each expansion step).

Antibody-secreting cells present in flasks were then cloned by limiting dilution (3.1.5).

3.1.5. Cloning cell lines

Aliquots of resuspended cells were counted (3.1.3.) and aliquots of the cells diluted into three different volumes of maintenance medium (3.1.2.), so that when dispensed in 100 μ l aliquots into 96-well plates, each individual well would contain an average of 1, 5, or 10 cells. The growing-up and screening procedures were repeated as for the initial growing stages after fusing except that maintenance medium was used throughout. (3.1.4.).

If a colony of antibody-secreting cells is produced from a single original cell then this is considered cloned and to be a true monoclonal antibody. The greatest problem is to avoid the presence of non-secreting cell lines which may have a tendency to outgrow their secreting neighbours. At each stage of expansion, it is

important to check that the cells are still secreting antibody as it is not uncommon for cells to stop secreting, especially in the initial expansion steps.

3.1.6. Freezing cells

Aliquots of established colonies of cells were frozen and stored for security. Approximately 1×10^6 cells were pelleted by centrifugation at 1000g for 10 minutes and quickly resuspended in 500 μ l of ice cold 10% DMSO (v/v) in foetal calf serum. The cells were initially stored at -70°C and then moved into liquid nitrogen for long term storage.

3.1.7. Screening cells

Initial screening of antibody-secreting cells was carried out on fixed tissue sections of bovine neural retina, unless otherwise stated (2.1.1.). From the 96-well plates, 20 μ l aliquots of culture medium from each of six adjacent wells were pooled and incubated on a single tissue section. The tissue sections were then subjected to treatment as described in Section 2.2.1. The six members of each group which showed anti-retinal antibody activity were then tested individually in order to identify which of the group of six contained the antibody-secreting cells.

3.2. Characterisation of antibody secreted by cell lines.

This was carried out by dot blotting, Western blotting and immune precipitation.

3.2.1. Dot blotting

2 μ l of the antigen-containing solution was dotted directly onto nitrocellulose membranes and allowed to dry. This dotting procedure was repeated several times in order to increase the concentration of each dot. The membranes were then quenched in TBS containing 0.2% (v/v) Tween 20. Antibody culture supernatant to be tested was diluted 1:5 in antibody-diluting solution (2.2.1.) and incubated with the nitrocellulose membrane overnight at room temperature. The nitrocellulose membrane was then washed in TBS containing 0.05 % (v/v) Tween 20 for 10 minutes and then in TBS for 5 minutes. This procedure was carried out between all subsequent incubation steps. The next step consisted of an incubation in antibody-diluting solution containing a 200-fold dilution each of sheep anti-mouse-IgG biotin conjugate and sheep anti-mouse-IgM biotin conjugate for 3 hours at room temperature. This was followed by a 1 hour incubation at room temperature with a streptavidin-HRP solution (Amersham International Plc), diluted 1000-fold in TBS containing 1mg/ml BSA. Peroxidase binding was visualised using the substrate 4-chloro-1-naphthol (2.2.2.).

3.2.2. Western blotting

The quenched nitrocellulose membrane from the Western blot (4.3.1.) was incubated overnight at room temperature with a 1:5 dilution of antibody culture medium in antibody-diluting solution (2.2.1.) and was then treated as for peroxidase staining of immunodots. (3.2.1.). substrate solution for 1 hour or until colour developed. The substrate was p-nitrophenyl phosphate

3.2.3. Enzyme-linked immunosorbent assay (ELISA)

The antigen was diluted in coating buffer (50mM sodium bicarbonate pH9.6) and 100 ul aliquots dispensed into the wells of a 96-well microtitre plate and incubated for 3-4 hours at room temperature. The wells were emptied and unoccupied binding sites quenched by the addition of 200 ul aliquots of coating buffer containing 0.5% (w/v) egg albumen. This was incubated at 4°C overnight before the wells were emptied. At this stage, the plates were either covered and stored at -20°C, or used immediately. If being used, the wells were washed three times with washing buffer (TBS containing 0.05% (v/v) Tween 20), each for 10 minutes. To each well was added either 100 ul of undiluted culture medium, culture medium diluted in antibody-diluting solution (2.2.1.), or partially-purified ascitic fluid (3.3.2.) diluted in antibody-diluting solution. The plate was incubated overnight at 4°C, the wells were washed as before and 100 ul of second antibody solution added and incubated, also overnight at 4°C. The second antibody solution was

antibody-diluting solution containing a 1000-fold dilution of either sheep anti-mouse-IgG alkaline phosphatase conjugate solution, or sheep anti-mouse-IgM alkaline phosphatase conjugate solution, depending on the antibody class of the monoclonal antibody. The wells were washed as before and then incubated with 100 μ l of substrate solution for 1 hour or until colour developed. The substrate was p-nitrophenyl phosphate disodium (1mg/ml) in 0.1M glycine pH10.4 containing 1mM magnesium chloride and 1mM zinc chloride. The reaction was stopped by the addition of 1N sodium hydroxide and the absorbance of the wells read at 405nm. Internal controls were carried out on each 96-well plate by the omission of all or one of the following:- antigen coat, monoclonal antibody solution or second antibody solution.

Absorbance readings were obtained for four replicate wells (treated identically) and the average taken. The average values obtained for the control wells were subtracted from the average values obtained for the experimental wells to allow for non-specific binding and substrate colouring.

3.2.4. Immune precipitations

This was carried out with the antigen used in its native form, or labelled with either biotin (4.6.3.1.), or 125 I-iodine (4.6.1.).

3.2.4.1. Immune precipitations using unlabelled antigen

This method was initially tested using a mouse monoclonal antibody against horseradish peroxidase.

The solid-state second antibody used was either sheep anti-mouse γ globulin coupled to Sepharose 4B (4.7.), or commercial goat anti-mouse IgM (μ chain-specific) conjugated to agarose beads.

1ml of the solid-state sheep anti-mouse γ globulin was incubated with 1.5ml of the monoclonal antibody culture medium for 3-4 hours. This was done in Eppendorf pots and spin-washing was done by pelleting the solid state antibody in washing buffer (TBS containing 0.05% (v/v) Tween 20) and resuspending the pelleted gel in fresh buffer. This was done four times before pelleting the gel and removing the buffer. To the pelleted gel was added 100 μ l of a 50 μ g/ml solution of horseradish peroxidase in TBS containing 1mg/ml of BSA. This was incubated at room temperature for 2 hours before spin washing as above. The binding of peroxidase to the gel was indicated by a colour change on the addition of the substrate 4-chloro-1-naphthol (2.2.2.) and the subsequent pelleting of the gel in this solution. The control gel, treated identically except that the monoclonal antibody had been omitted, remained colourless.

3.2.4.2. Immune precipitations using labelled antigen

Antigen was labelled by means of ^{125}I (4.6.1.), or by biotin (4.6.3.) and the same procedure carried out as above.

The pelleted gel containing the monoclonal antibody and the antigen mixture was incubated with sample incubation buffer (60°C) (4.1.1.) for 20 minutes and this was subjected to polyacrylamide gel electrophoresis (4.1.1.). In the case of the ^{125}I -labelled antigen, autoradiography was carried out (4.2.4.) and in the case of the biotin labelled antigen, the gel was Western blotted (4.3.1.) and stained to visualise biotinylated proteins by incubating the nitrocellulose with streptavidin-HRP (4.3.3).

3.3. Ascitic fluid

Monoclonal antibody culture media generally contain antibody concentrations of 1-10 $\mu\text{g}/\text{ml}$. In order to produce larger quantities of antibody, ascitic fluid may be produced containing up to 10mg/ml of antibody.

3.3.1. Production of ascitic fluid

Ten week-old female Balb/c mice were injected intraperitoneally with 100 μl aliquots of Pristane (2,6,10,14-tetramethylpentadecane) and one month later with 1×10^6 antibody-secreting cells which had been

pelleted and resuspended in 500 ul of physiological saline. Tumours were allowed to form over the next four weeks before the ascitic fluid was drained.

The ascitic fluid was allowed to settle on standing for 2 hours before the upper layer was removed from the lower layer of cells. The upper layer was aliquoted and stored at -20°C.

3.3.2. Purification of ascitic fluid

The ascitic fluid was tested for antibody content using fixed tissue sections of bovine neural retina (2.1.1.), using the immunofluorescence method (2.2.1.).

Purification steps were tested by the same method.

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3.3.2.1. Purification of ascitic fluid using DEAE cellulose ion exchange chromatography (4.8.3.)

5ml of swollen gel (DE52, Whatman Ltd, Maidstone, Kent) was equilibrated in 50mM Tris HCl pH 8.4. 1.5ml of ascitic fluid (dialysed against the same buffer) was added to the column. More of the same buffer was added and 0.5ml fractions collected. The optical density at 280nm was measured to determine protein content and antibody content tested (3.3.2.).

Those fractions with both a high optical density and specific-antibody content were retained and stored in aliquots at -20°C.

Electrophoretic methods.

SDS-Polyacrylamide gel electrophoresis

Unless otherwise stated SDS-PAGE was carried out using a discontinuous buffer system (Laemmli and Favre 1973) with a 10% acrylamide separating gel and a 5% acrylamide stacking gel.

The sample was prepared by adding it to half its volume of sample reduction buffer, consisting of 0.2M Tris HCl pH 6.8 containing 5% SDS (w/v), 0.4M EDTA and 40% glycerol (v/v). The mixture was heated to 100°C and

boiled for 10-20 minutes before a small drop of

SECTION 4: GENERAL METHODS AND MATERIALS.

Laemmli's sample tracking dye was added (approx 1mg/ml) and the mixture loaded onto the gel.

The gel was run until the tracking dye was approximately 1/3 from the foot of the gel. This was done either overnight with a constant voltage (40 volts), or quickly using the day with a constant current (40 milliamps).

SDS polyacrylamide gel electrophoresis separates proteins on the basis of their molecular weights (Laemmli et al 1967). In general over a wide range of molecular weights there is a linear relationship between the molecular weight of the protein and its R_F value (Laemmli et al 1967; Weber and Osborn 1969). This relationship can be used to estimate the molecular

4.1. Electrophoretic methods.

4.1.1. SDS-Polyacrylamide gel electrophoresis

Unless otherwise stated SDS-PAGE was carried out using a discontinuous buffer system (Laemmli and Favre 1973) with a 10% acrylamide separating gel and a 5% acrylamide stacking gel.

The sample was prepared by adding it to half its volume of sample incubation buffer, consisting of 0.2M Tris HCl pH 6.5 containing 8% SDS (w/v), 8mM EDTA and 40% glycerol (v/v). The mixture was heated to 60°C and incubated for 15-20 minutes before a small drop of bromophenol blue tracker dye was added (approx 1mg/ml) and the mixture loaded onto the gel.

The gel was run until the tracker dye was approximately 1cm from the foot of the gel. This was done either overnight with a constant voltage (40 volts), or quickly during the day with a constant current (40 milliamps).

SDS-polyacrylamide gel electrophoresis separates proteins on the basis of their molecular weights (Shapiro et al 1967). In general over a wide range of molecular weights there is a linear relationship between log molecular weight of the protein and its R_f value (Shapiro et al 1967; Weber and Osborn 1969). This relationship can be used to estimate the molecular

weight of proteins of unknown mass by using a number of well characterised marker proteins of known molecular weight to calibrate each gel.

4.1.2. 2-Dimensional gel electrophoresis

This was carried out according to the method of O'Farrell (O'Farrell 1975).

In the first dimension a pH gradient was established on running the gel after loading the sample. The gel was run under denaturing and reducing conditions. To the sample was added a quarter of its volume of sample buffer containing 9.5M urea, 5% Triton X100 (v/v), 7% ampholytes (v/v) and 7% β -mercaptoethanol (v/v).

The sample was loaded onto the top of the gel, in contact with the top gel buffer (0.4% ethanolamine (v/v)) and a current was established between this and the bottom buffer (0.2% ortho-phosphoric acid (v/v)). The pH gradient formed across the gel was measured by incubating slices of the gel after electrophoresis in boiling water for 10 minutes, and measuring the pH of the solution using pH indicator paper.

In the second dimension a 6-15% exponential acrylamide gradient separating gel and 5% acrylamide stacking gel were used.

This was run as described in Section 4.1.1. This mixture when made up to 100ml with distilled water, constituted the stain solution. The washed gel was incubated in fresh stain solution for 20 minutes at room

4.2. Staining of electrophoresis gels

4.2.1. Coomassie Blue staining of protein.

Slab gels were fixed for 1hr at room temperature in a 20% methanol (v/v), 10% acetic acid (v/v) solution. Staining was then done in Coomassie Brilliant Blue R stain solution (0.25% (w/v) Coomassie Brilliant Blue R-250 in 25% (v/v) methanol, 10% acetic acid, (v/v)) for 20 minutes at room temperature before destaining in destain solution (10% methanol, 7% acetic acid (v/v)) containing small squares of white polyurethane foam to absorb free dye in the solution.

4.2.2. Silver staining of protein.

This is a much more sensitive protein stain than the Coomassie blue stain and can detect protein at the 1ng level. This was carried out according to the method of Wray et al (Wray et al 1981). The slab gel was fixed for 1hr in a 40% methanol (v/v), 10% acetic acid (v/v) solution. It was then soaked in 50% methanol (v/v) for 1hr, followed by 10 minutes in distilled water. This methanol/water routine was repeated twice. Solution A, containing 0.6g of silver nitrate in 4ml of distilled water was added dropwise to a continuously stirred solution B. Solution B contained 21ml of 0.36% sodium hydroxide (w/v) and 1.4ml of 14.8M ammonium hydroxide. This mixture when made up to 100ml with distilled water, constituted the stain solution. The washed gel was incubated in fresh stain solution for 20 minutes at room



temperature. The gel was then washed in distilled water for 20 minutes with changes every 5 minutes. The developing solution was prepared by mixing 2.5ml of 1% (w/v) citric acid and 250 μ l of 38% (v/v) formaldehyde followed by the addition of 500ml of distilled water. The developing solution was used immediately to develop the protein stains. This process was halted by replacing the developing solution with a 10% (v/v) acetic acid solution and the stained gel was stored in 50% (v/v) methanol.

4.2.3. Periodic acid-Schiff's staining of carbohydrate.

This is a general carbohydrate stain of relatively low sensitivity used in polyacrylamide gels. It was carried out according to the method of Zacharius (Zacharius et al 1969).

The gel was initially soaked in 12.5% (w/v) trichloroacetic acid for 30 minutes, rinsed in distilled water and soaked in a fuschin sulphite solution in the dark for 50 minutes and then washed three times, each for 10 minutes, in a 0.5% (w/v) potassium metabisulphite solution. Finally the gel was washed in distilled water until it became colourless, when it was transferred into, and stored in 5% (v/v) acetic acid.

4.2.4. Autoradiography of polyacrylamide gels.

The polyacrylamide gel was fixed, stained and destained (4.2.1.) and then dried down under vacuum onto blotting paper. This was then exposed to a FUJI X-ray film and stored in an X-ray cassette at -70°C prior to the film being developed.

4.3.1. Western blotting.

Proteins were transferred from slab gels according to the method of Towbin (Towbin 1979). A 0.5A current was used for two hours. Amido black stain was used to test for the transfer of protein from the gel to the nitrocellulose. (4.3.2.) After transfer, quenching of the unoccupied binding sites of the nitrocellulose membranes was carried out by a 1 hour incubation in TBS containing 0.2% TWEEN 20 (v/v). The membrane was then treated with antibody (3.2.2.), or lectin (4.3.3.).

4.3.2. Amido black staining of protein

This is a general protein stain of low sensitivity used to stain nitrocellulose. It was carried out according to the method of Davies (Davies 1964).

The nitrocellulose from 4.3.1. was stained for 2-3 minutes in amido black before quenching and then destained in destain solution (90% methanol (v/v), 2% acetic acid (v/v)).

4.3.3. Lectin treatment of Western blots

The quenched nitrocellulose was incubated for 2 hours at room temperature in a 100-fold dilution of stock lectin-biotin conjugate (4.6.3.2.), in TBS containing BSA (1mg/ml,w/v). The nitrocellulose was then rinsed for 10 minutes in TBS/0.05% TWEEN 20 (v/v) followed by 5 minutes in TBS, before the addition of Streptavidin-HRP conjugate diluted 1000-fold in TBS containing BSA (1mg/ml,w/v). This was incubated at room temperature for 1 hour before visualising HRP binding by the addition of the substrate 4-chloro-1-naphthol (2.2.2). The alternative method was to incubate the nitrocellulose for 1 hour at room temperature in a fifty-fold dilution of peroxidase-lectin conjugate prior to chloronaphthol staining.

4.3.4. Lectin treatment of dot blots

This was carried out as for lectin treatment of Western blots (4.3.3.) except that the sample being tested was dotted directly onto the nitrocellulose before quenching (4.3.1).

4.4. Bradford protein assay.

This was carried out according to the method of Bradford (Bradford 1976).

Aliquots of the protein solutions were each added to 0.5ml aliquots of Bradford reagent containing 0.01% Serva blue dye (w/v), 4.7% ethanol (v/v) and 8.5% phosphoric acid (v/v). The mixtures were allowed to stand for 10-15 minutes before the absorbance at 595nm was read and interpreted by means of a set of standard (0 - 100 ug/0.5ml) BSA solutions.

4.5. Immunoglobulin isolation from serum

A 5ml aliquot of sheep serum containing anti-mouse immunoglobulin was added to 10ml of 50mM sodium acetate pH4.0. 370 ul of octanoic acid was added drop-wise to the mixture which was stirred continuously. This was left to stand for 30 minutes at room temperature before the precipitate was pelleted by centrifuging the mixture at 100 000g for 30 minutes. The resulting soluble globulin fraction was dialysed against the appropriate buffer overnight and used as required.

4.6. Labelling of protein

4.6.1. Labelling with ^{125}I using the Chloramine T method.

This was carried out according to the method of Greenwood et al 1963. 200 ul of sample containing 0.2mg of soluble protein was dialysed against 50mM phosphate buffer pH7.2 containing 0.15M sodium chloride. This was mixed with 10 ul (0.5mCi) of sodium ^{125}I iodide and 50 ul of a fresh 16mg/ml chloramine T

solution. This was left for 2 minutes at 0°C before 50 ul of 19mg/ml sodium thiosulphate and 25 ul of 30mg/ml sodium iodide solutions were added. The mixture was then desalted to remove free iodide using a P6DG desalting gel (4.8.2.). A 10 ul sample of the labelled fraction was counted in a gamma counter to check the efficiency of ^{125}I incorporation.

4.6.2. Protein labelling with FITC.

4.6.2.1. Streptavidin

0.5mg of lyophilised streptavidin was dissolved in 100 ul of distilled water and added to 400 ul of 50mM sodium bicarbonate buffer pH 9.5. To this was added 20 ul of a 10mg/ml (w/v) solution of FITC (isomer II) in dimethyl sulphoxide (DMSO). This mixture was stirred in the dark for 4-5 hours. A volume of ethanolamine pH8 was added to give a final 50mM solution. This was incubated for 30 minutes and then dialysed overnight at 4°C against TBS.

4.6.2.2. Lectins

This was carried out as for streptavidin, using a 1mg/ml (w/v) lectin solution containing the hapten sugar at a concentration of 200mM.

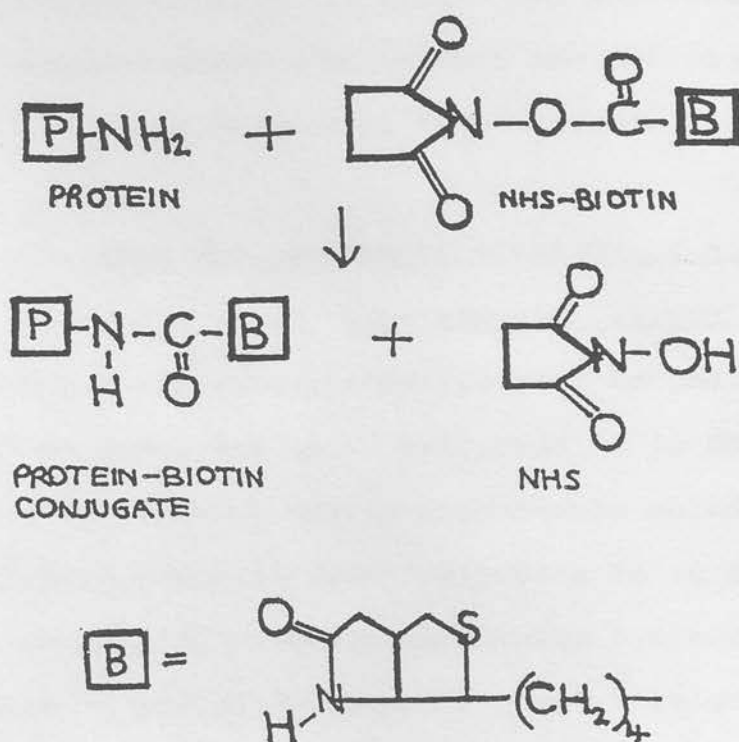


Figure 4.1. The reaction mechanism involved in coupling biotin to protein using NHS-biotin. The nucleophilic, unprotonated ϵ -amino groups of the lysine residues of the protein molecules attack the activated ester of NHS-biotin resulting in an amide bond and the release of N-hydroxy-succinimide. See text for experimental detail.

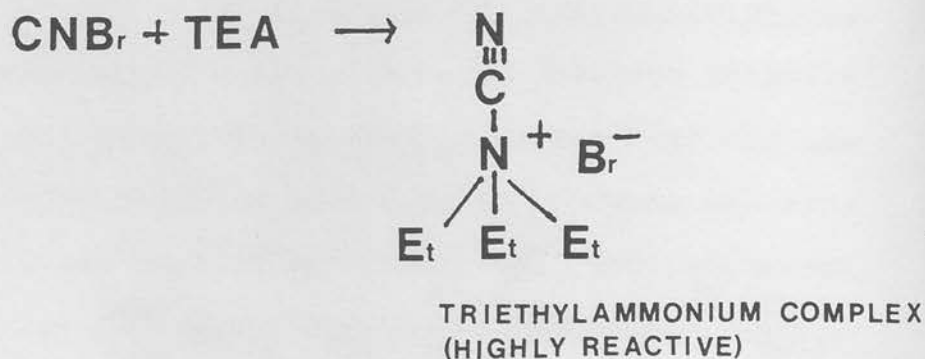
4.6.3.1. Protein Labelling with N-Hydroxy-succinimidobiotin (NHS-Biotin).

A 1mg/ml solution of protein was dialysed overnight against 50mM sodium bicarbonate buffer pH9.5. To 1ml of this was added 20 ul of a 33mg/ml NHS-biotin in dimethyl formamide (DMF) solution. The mixture was stirred for 1 hour at room temperature before dialysis against TBS overnight (Figure 4.1.).

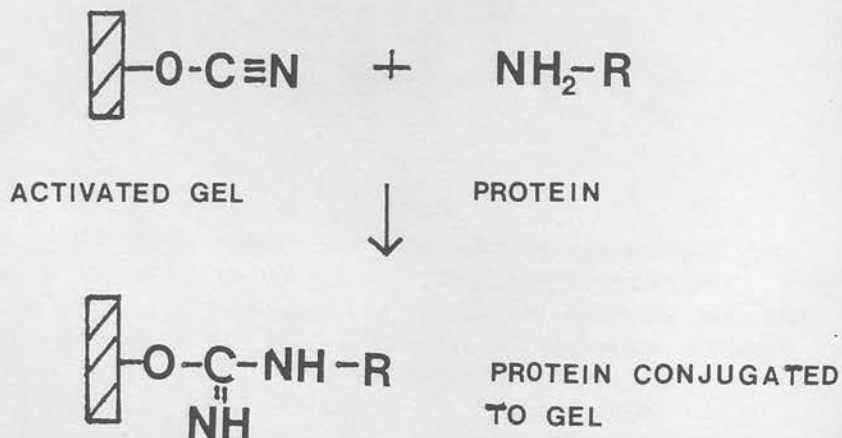
4.6.3.2. Lectins

This was carried out as above using a 1mg/ml solution of lectin containing the hapten sugar at a concentration of 200mM.

A)



B)



CNBr = cyanogen bromide
TEA = triethylamine
HBr = hydrogen bromide

Figure 4.2 The reaction mechanisms involved in:
A. activation of Sepharose 4B by cyanogen bromide in the presence of triethylamine.
B. coupling of the activated Sepharose 4B to protein.

4.7.1. Cyanogen bromide activation of Sepharose 4B (Kohn and Wilchek 1982) (Figure 4.2.).

15g of Sepharose 4B was sequentially washed in distilled water, 30% acetone, 60% acetone solutions and then resuspended in a 60% acetone solution. This was cooled to -15°C on a salt/ice mixture and all subsequent steps and reagents were maintained at this low temperature. 375mg of cyanogen bromide dissolved in 375 μl of acetone and 375 μl of 1.5M triethylamine in acetone were added with constant stirring. The activated gel was quickly added to 150ml of a 1:1 mixture (v/v) of acetone and 0.1M HCl. The gel is in a stable form at this low pH. Rehydration was carried out by rapid sequential washes in 60% acetone, 30% acetone and 50mM sodium bicarbonate buffer pH 9.5 before the gel was resuspended in 15ml of the buffer solution.

4.7.2. Coupling of protein to cyanogen bromide-activated Sepharose 4B

The protein to be coupled to the activated gel was dialysed against 50mM sodium bicarbonate buffer pH9.5. To each ml of activated gel mixture was added one of the following:- 1ml of sheep anti-mouse γ globulin (4.5.), or 1ml of a 1mg/ml lectin solution containing the hapten sugar at a concentration of 200mM. The mixture was incubated overnight on an end-over-end mixer at 4°C . The gel was washed in TBS, or column buffer in the case of the lectin-conjugated gel

(4.8.1.). The protein content of the samples before and after addition to the activated gel were compared using the Bradford assay (4.4.) to check the efficiency of coupling.

4.8. Chromatography methods

4.8.1. Lectin affinity chromatography

This was carried using either a Sepharose 4B gel coupled to lectin (4.7.2.) or commercial lectin-conjugated agarose. The sample to be chromatographed was dialysed against column buffer and all further steps were done in the presence of either column buffer or eluting buffer. The column buffer was 0.1M HEPES/sodium hydroxide pH7.2 containing 0.15M sodium chloride, 0.05% sodium azide (w/v), 1mM calcium chloride, 1mM magnesium chloride and 1mM manganese chloride. The eluting buffer had the addition of the hapten sugar at a concentration of 200mM for the appropriate lectin conjugate being used. 1ml of sample (1mg/ml of protein) was run into, but not through, the lectin-agarose (3ml bed volume) and incubated for 2 hours at room temperature. The gel was washed through with column buffer to remove all unbound material. The eluting buffer was then run into the gel and incubated for 2 hours at room temperature. Eluted material was collected when subsequent additions of one bed volume of eluting buffer were made. When elution

was complete the column was stored in eluting buffer at 4°C. The Bradford protein assay was used to check the washing and eluting steps were complete. In some cases it was found easier to carry out this procedure in batch form where the sample was mixed end-over-end with a suspension of the gel. Washing and eluting steps were carried out by a series of spin washes of the gel mixture and the appropriate buffer (2500 rpm for 10 minutes).

4.8.2. P6DG gel filtration (desalting) chromatography

This method was used as a quick alternative to an overnight dialysis step. 1ml aliquots of swollen gel (P6DG BioRad Laboratories, Richmond, California) were washed in TBS prior to 200 ul of sample being loaded and run into the gel. In some cases an unreactive, coloured dye (phenol red) was added to the sample prior to loading, to indicate the position of the low molecular weight material within the gel. Fractions were collected up to the point where the dye was eluted, and tested for protein content by the Bradford method (4.4.). Those containing protein were pooled and stored to be used as required.

The alternative method used was to add 0.5ml aliquots of swollen gel to Eppendorf pots into which small holes had been previously pin-pricked at the top and bottom, and with small amounts of glass wool placed inside the bottom. After spin-washing with TBS (by centrifuging at

1000g for 10 minutes and replacing the supernatant with fresh TBS), the gel was finally spun dry. To this was added 0.5ml of sample which was then centrifuged as above and the eluted material collected from the Eppendorf pot-holder.

4.8.3. DE cellulose ion exchange chromatography

Swollen gel (DEAE 52 cellulose, Whatman Ltd, Maidstone, Kent) was equilibrated in 25mM Tris HCl buffer pH7.2 and packed into a column (3ml bed volume). Samples were dialysed against the same buffer before application to the column. 0.5ml of sample (1mg/ml) was added to the gel and unbound material was removed by the further addition of the same buffer. Elution of bound material was by the addition of aliquots of sodium chloride in the Tris buffer, each aliquot increasing in concentration in increments of 50mM. Care was taken to ensure that the elution of material by a particular salt concentration was complete before the salt concentration was increased. The protein content of the fractions was checked by the use of the Bradford assay (4.4.). The alternative batch method was used at times as for the affinity chromatography (4.8.1.).

4.8.4. Fast protein liquid chromatography using the Superose 12 HR 10/30 gel filtration FPLC Column.

The Superose 12 column (24ml bed volume), was equilibrated with TBS before 500 μ l of sample (1mg/ml of protein) was loaded. A flow rate of 0.3ml/minute was maintained and 0.5ml fractions collected. Protein in the eluate was detected by the absorbance at 280nm and information recorded on a chart recorder. The molecular weights of the proteins eluted from the column were calculated by comparison with the retention times of proteins, of known mass.

Control incubations were carried out without the enzyme.

4.8.5. Neuraminidase treatment of glycoproteins

Fixed tissue sections were rinsed for ten minutes in 100mM TBS pH5.5 before being incubated for thirty minutes in 100 μ l of the same buffer containing 0.1U of neuraminidase (type V from Clostridium perfringens). The tissue section was then rinsed in TBS and treated with antibody or lectin as required.

4.9. Enzymatic Treatments

4.9.1. Trypsin treatment of protein

The sample containing protein was incubated in TBS containing 10U/ml of bovine pancreatic trypsin. The reaction was stopped by the addition of phenyl-methylsulphonyl fluoride (PMSF) to a final concentration of 0.1mM.

Fixed tissue sections to be treated with the enzyme were rinsed for ten minutes in TBS before being incubated for ten minutes at room temperature in 100ul of the same buffer containing 1U of the enzyme. The tissue section was then well rinsed with TBS and treated with antibody as required.

Control incubations were carried out without the enzyme.

4.9.2. Neuraminidase treatment of glycoconjugates.

Fixed tissue sections were rinsed for ten minutes in 100mM MES pH5.0 before being incubated for thirty minutes in 100ul of the same buffer containing 0.1U of neuraminidase (type V from *Clostridium perfringens*). The tissue section was then rinsed in TBS and treated with antibody or lectin as required.

4.9.3. Heparinase treatment of proteoglycan

The sample containing proteoglycan was incubated with heparinase II (from *Flavobacterium heparinum*) (0.2U/ml final concentration) in 50mM sodium acetate pH 5.0 containing 1mM calcium chloride, overnight at room temperature.

Fixed tissue sections were rinsed for ten minutes in 100mM sodium acetate pH5.0, containing 1mM calcium chloride and were then incubated for five hours at room temperature in the same buffer containing 0.25U of the enzyme. The sections were then rinsed with TBS and treated with antibody as required. Control incubations were carried out without the enzyme.

4.9.4. Chondroitinase ABC treatment of proteoglycan.

50 ul of the sample containing proteoglycan was added to an equal volume of chondroitinase ABC (from *Proteus vulgaris*) (in distilled water) to give a final concentration of 0.5U/ml. The mixture was incubated for 2 hours at 37°C.

Fixed tissue sections were rinsed for ten minutes in TBS before being incubated for five hours at room temperature in 100ul of the same buffer containing 0.1U of the enzyme. The sections were then rinsed in TBS and treated with antibody as required.

Control incubations were carried out without the enzyme.

4.9.5. Nitrous acid treatment of glycosaminoglycan

The sample containing glycosaminoglycan was incubated in 1.8M acetic acid containing 0.25M sodium nitrite (final concentration) for 2 hours at 37°C. This was then dialysed against TBS.

4.9.6. Chondroitinase AC treatment of proteoglycan

Fixed tissue sections were rinsed for ten minutes in TBS before being incubated for five hours at room temperature with 500ul of the same buffer containing 0.5U of the enzyme. The tissue sections were then rinsed in TBS before being treated with antibody as required.

4.9.7. Hyaluronidase (testicular) treatment of proteoglycan

The sample containing proteoglycan was incubated with 50mM sodium acetate pH5.0 containing 300U/ml type 1-S bovine testes hyaluronidase, for 2 hours at 37°C.

Fixed tissue sections were rinsed for ten minutes in 100mM sodium acetate pH5.0 before being incubated for five hours at room temperature with 500ul of the same buffer containing 150U of the enzyme. The section was then rinsed in TBS before being treated with antibody as required.

Control incubations were carried out without the enzyme.

4.9.8. Hyaluronidase (Streptomyces) treatment of

proteoglycan. were obtained from BDH Chemicals Ltd and

Fixed tissue sections were rinsed for ten minutes in

100mM sodium acetate pH5.0 before being incubated for

five hours at room temperature with 100ul of the same

buffer containing 500U of hyaluronidase (from Streptomyces were

Streptomyces hyalurolyticus). The section was then

rinsed with TBS before being treated with antibody as

required. Ilex Ltd, Irvine and ELISA plates from Falcon

Laboratory, Oxford.

Cattle eyes were obtained from the local slaughterhouse

and mice from animal house stock.

The FPLC columns and amphotytes were obtained from

Pharmacia Ltd, Milton Keynes.

Nitrocellulose membranes (0.45 um, 400 x 200mm) were

obtained from Schleicher and Schuell, Dassel, W Germany.

¹²⁵I-Sodium Iodide was supplied by the Radioimmunoassay

Department, University of Edinburgh.

Tissue Tak OCT was obtained from Lab-Tek Products,

Division Miles Laboratories Inc.

4.10. Materials

All chemicals were obtained from BDH Chemicals Ltd and were of AnalaR grade, or from Sigma, London, unless otherwise indicated in the text.

All enzymes, commercial antibody solutions, lectin solutions and tissue culture media and supplements were obtained from Sigma, London.

Tissue culture plastic was obtained from Flow Laboratories Ltd, Irvine and ELISA plates from Falcon Laboratory, Oxford.

Cattle eyes were obtained from the local slaughterhouse and mice from animal house stock.

The FPLC columns and amphylytes were obtained from Pharmacia Ltd, Milton Keynes.

Nitrocellulose membranes (0.45 μ m, 400 x 200mm) were obtained from Schleicher and Schuell, Dassel, W Germany.

125 I-Sodium Iodide was supplied by the Radioimmunoassay Department, University of Edinburgh.

Tissue Tek OCT was obtained from Lab-Tek Products, Division Miles Laboratories Inc.

5.1. Preparation of Interphotoreceptor Matrix (IPM)

Retinal eyes were collected from the slaughterhouse and transported on ice. The eyes were then immediately cut and vitellineous membranes were removed. The eyes were then cut into halves and all solutions and tissues were maintained at 4°C. The eyes were rinsed free of excess fatty tissue, cut in half, the lens and vitreous removed and the anterior portion discarded.

The retina was rinsed in 1-2 ml of TBS (containing 0.1mM EDTA) in order to separate it from the adjacent layer of retinal pigmented epithelium (RPE) cells. The retina was then cut off by cutting it from the optic nerve and was

SECTION 5: TISSUE PREPARATION. This process was repeated with each eye, using the same TBS rinsing solution to produce a highly concentrated solution of IPM in a small volume. The rinsing solution was centrifuged at 100,000g for 30 minutes, the soluble fraction was removed, aliquoted into smaller volumes and stored at -20°C.

The protein content of the IPM was estimated by the Bradford method (4.4) and was usually 1-2mg/ml, depending on the number of retinas available and the volume of TBS used.

5.2. Preparation of interstitial retinol binding protein (IRBP)

IRBP was purified from IPM by ConA-affinity chromatography (4.8.1.) using either a commercial ConA-Agarose gel, or ConA coupled to Sepharose 4B gel by the cyanogen bromide activation method (4.7.1 and 4.7.2.). The Sepharose 4B-ConA gel was estimated to contain approximately 1mg of lectin per ml of swollen gel, therefore a ratio of 1 volume of gel to 2 volumes of a 1mg/ml solution of IPM was used to remove all the IRBP from a volume of IPM. This assumes that at least two of the four mannose binding sites on each of the ConA molecules are still active after coupling to the gel and that ConA and IRBP have approximately the same molecular weight.

The commercial gel contains (according to the suppliers) 10-20mg of lectin/ml of gel. Small volumes of the gel were mixed with ten times its volume of Sepharose 4B to make handling easier.

2ml of the ConA-agarose gel or the ConA-agarose/Sepharose 4B mixture was equilibrated in column buffer (4.8.1). The gel was centrifuged at 1000g for 10 minutes, the supernatant replaced with fresh column buffer and the process repeated twice. The gel was finally pelleted before the addition of 4ml of IPM (1mg/ml), previously dialysed against column buffer overnight. This was mixed end-over-end for 3-4 hours before spin washing the gel as above. The first

supernatant was collected and called "IPM-IRBP" (in some cases this fraction was subjected to the above treatment for a second time in order to deplete the IPM of IRBP completely). To the pelleted gel was then added 2ml of column buffer containing 200mM α -methyl mannoside. The mixing and spin washing procedures were repeated. This time, the first two supernatants, containing the majority of the eluted IRBP, were collected. The fractions were tested for protein content by the Bradford assay (4.4.) and for purity by SDS-polyacrylamide gel electrophoresis (4.1.1.) followed by treating subsequent Western blots with ConA (Figures 5.1. and 5.2.).

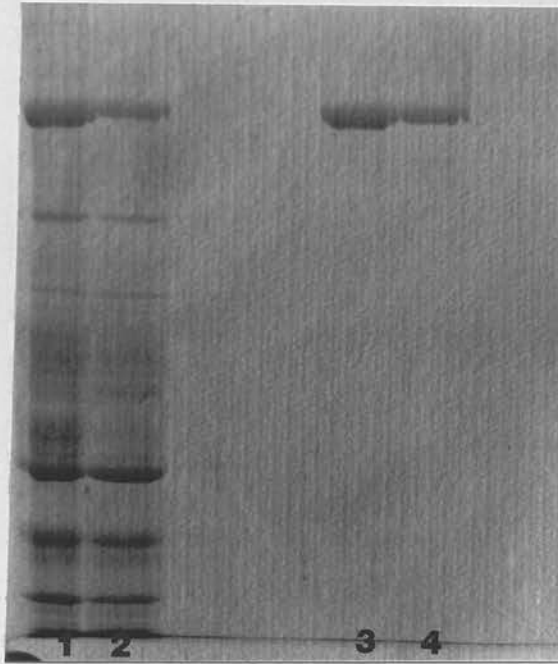


Figure 5.1 10% polyacrylamide gel stained with Coomassie blue showing the purification of IRBP from IPM by ConA affinity chromatography. See text for experimental detail.

1. IPM (100ug) (Starting material).
2. IPM depleted of IRBP (1st pass-through fraction).
3. IRBP (1st purified fraction).
4. IRBP (2nd purified fraction).

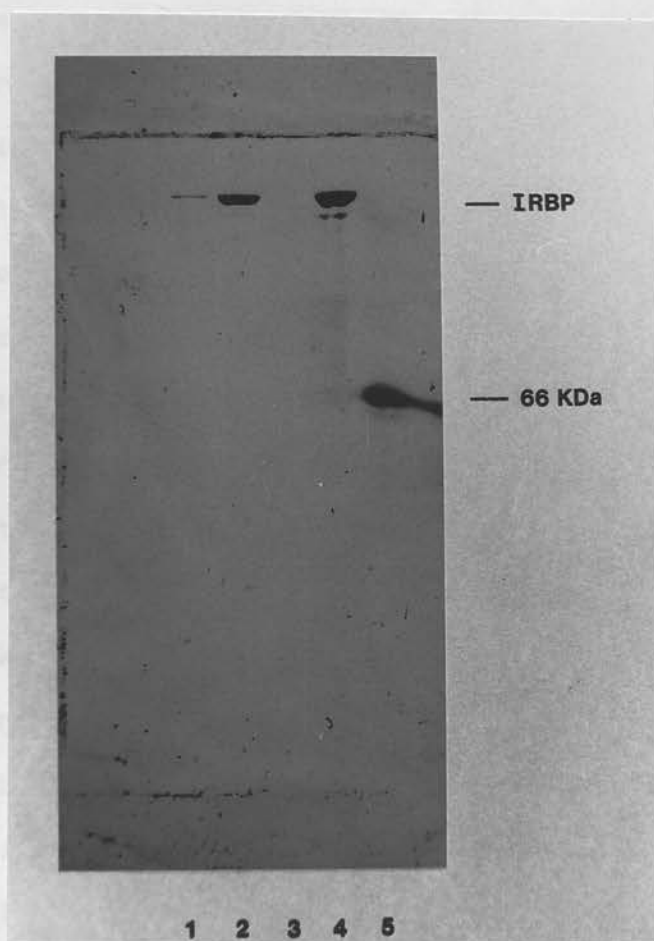


Figure 5.2. Western blot of a 10% polyacrylamide gel treated with ConA-bio, followed by Streptavidin-HRP and stained with chloronaphthol, showing the purification of IRBP from IPM by ConA affinity chromatography. See text for experimental detail.

1. IRBP (2nd purified fraction).
2. IRBP (1st purified fraction).
3. IPM depleted of IRBP.
4. IPM (100ug) (Starting material).
5. Bovine serum albumin.

5.3. Preparation of crude photoreceptor outer segments (PROS)

The eyecup was treated as for IPM, the retina in this case being separated from the RPE layer in 50mM Tris HCl pH7.4 containing 0.32M sucrose and 0.1mM PMSF. The retina was added to more of the same solution when detached from the eyecup. Approximately 1-2ml of solution was allowed for each eye. The retinae were lightly homogenised in a loose-fitting teflon-glass homogeniser and then centrifuged at 1000g for 10 minutes. The supernatant was removed and the top layer of the pellet was collected. (This was pinkish in colour indicating the presence of the pigment proteins from the outer segments of the photoreceptors). The pellet was resuspended in the sucrose buffer containing one tenth of its volume of stock 10% Triton X100 detergent solution (final 1% solution)(v/v). The solution was stood on ice for 30 minutes before being centrifuged at 100 000g for 30 minutes. The soluble fraction was removed, aliquoted into smaller volumes and stored at -20°C. (Figure 5.3.).

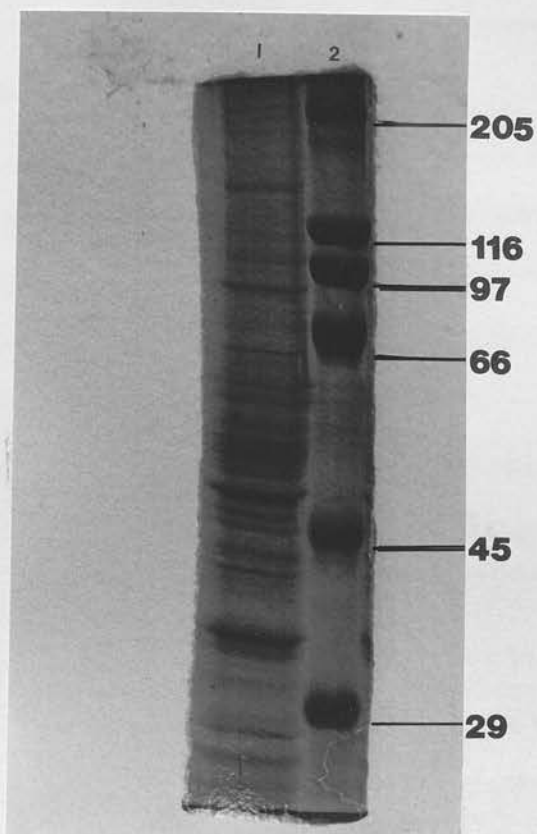


Figure 5.3. 10% polyacrylamide gel stained with Coomassie blue. See text for experimental detail.

1. crude photoreceptor outer segments (80ug).
2. molecular weight markers.

6.1. Introduction

The aim of this project was to produce monoclonal antibodies which would react specifically with cell surface molecules of the photoreceptor cells, or with molecules present in the interphotoreceptor matrix (IPM) which are associated with the surfaces of the photoreceptors.

This would enable such cell surface or cell surface associated molecules to be identified and characterised biochemically. Comparisons could then be made between normal adult tissue, developing tissue and diseased tissue.

A monoclonal antibody raised against a molecule specific to a single cell SECTION 6: RESULTS. used to identify other constituents of the same cell type, which may co-purify with the antigen. Therefore monoclonal antibodies raised against cell-specific molecules may be very powerful tools in the elucidation of molecular constituents of a specific cell.

Of particular value in a study of the photoreceptor cells and the IPM would be monoclonal antibodies which differentiate between the disc membrane and the plasma membrane of the rod photoreceptor cells. Much work has been done biochemically on the discs of the rod photoreceptor cells but less is known about the plasma membrane of these cells. This is largely because the plasma membrane of the rod photoreceptors comprises only 2% of the total membrane content of the cells, the

6.1. Introduction

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This would enable such cell surface or cell surface associated molecules to be identified and characterised biochemically. Comparisons could then be made between normal adult tissue, developing tissue and diseased tissue.

A monoclonal antibody raised against a molecule specific to a single cell type may also be used to identify other constituents of the same cell type, which may co-purify with the antigen. Therefore monoclonal antibodies raised against cell-specific molecules may be very powerful tools in the elucidation of molecular constituents of a specific cell.

Of particular value in a study of the photoreceptor cells and the IPM would be monoclonal antibodies which differentiate between the disc membrane and the plasma membrane of the rod photoreceptor cells. Much work has been done biochemically on the discs of the rod photoreceptor cells but less is known about the plasma membrane of these cells. This is largely because the plasma membrane of the rod photoreceptors comprises only 2% of the total membrane content of the cells, the

majority being present in the disc membranes. Monoclonal antibodies may be used to identify molecules common to both types of membranes, or molecules specific for the plasma membrane.

Monoclonal antibodies which specifically recognise the cone photoreceptors would also be very useful. To date there is no method of isolating the cone outer segments from the retina. The production of monoclonal antibodies which have immunoreactivity against these cells would seem a reasonable approach to identifying some of their cellular components.

Such monoclonal antibodies may allow the identification of constituents of the rod and cone cells which are specific to a single cell type, or which are common to rods and cones, and would also allow the two cell types to be distinguished unequivocally. Further biochemical analysis of these molecules would then be possible, and their appearance through development and in diseased tissue may be monitored.

Three methods of approaching the problem of producing monoclonal antibodies specific for cell surface, or cell surface associated molecules of the photoreceptor cells were taken.

1. To immunise mice repeatedly with unpurified preparations of antigen mixtures for example crude photoreceptor outer segments (5.3.), or IPM (5.1.), and to carry out many cell fusions, selecting hybridoma cell lines which secrete antibodies that react specifically

with the IPM or the photoreceptor cells. This approach relies heavily on luck and there is no guarantee of producing antibodies which are specific to any particular cell type.

2. To purify, or at least partially purify some of the constituents known to be present either within, or associated with, the surfaces of the photoreceptor cells, for example, the PNA-binding material associated with the cone photoreceptors (Blanks and Johnson 1984). Such relatively pure fractions can be used to immunise mice. There should be a higher probability of any monoclonal antibodies produced from subsequent fusions being immunoreactive with either the rod or cone surfaces, compared to any antibodies produced by method 1. above. This more selective immunisation process should increase the chances of producing the required cell-specific monoclonal antibodies.

3. To synthesise a protein, or a peptide fragment of a protein, which is known to be unique to a single retinal cell type (for example a cone-specific protein) and to use this as the immunogen in mice prior to cell fusion. Monoclonal antibodies produced by this method should be reactive with a single protein and therefore with the single cell type in which the protein is found. The monoclonal antibody may then be used to identify the cell type and also possibly to isolate further constituents of the same cell type for further analyses.

The above three methods were used in attempts to raise monoclonal antibodies specific for the surfaces of the rod and cone photoreceptor cells, or against material in the IPM which is associated with the photoreceptor cells.

This was carried out using bovine tissue (available in abundance) as the antigen source, with a view to transferring to human and/or mouse tissue systems (less readily available in large quantities) for further studies using the antibodies produced against the bovine retina.

6.2. Repeated immunisation of mice with unpurified antigen mixtures

This was the first approach taken to solve the problem of obtaining cell specific monoclonal antibodies.

Mice were immunised (3.1.1.) with 1mg/ml solutions of either IPM (5.1.) or crude photoreceptor outer segments (5.3.). The subsequent cell fusions were carried out by Dr J Haywood (3.1.4.) and antibody-secreting cell lines were identified, using fixed tissue sections of bovine neural retina (3.1.7.) which were treated to visualise antibody binding by the immunofluorescence method (2.2.1.). Those cell colonies identified as secreting anti-retinal antibodies were expanded (3.1.4.) and finally cloned (3.1.5.) to produce true monoclonal antibody-secreting cell lines.

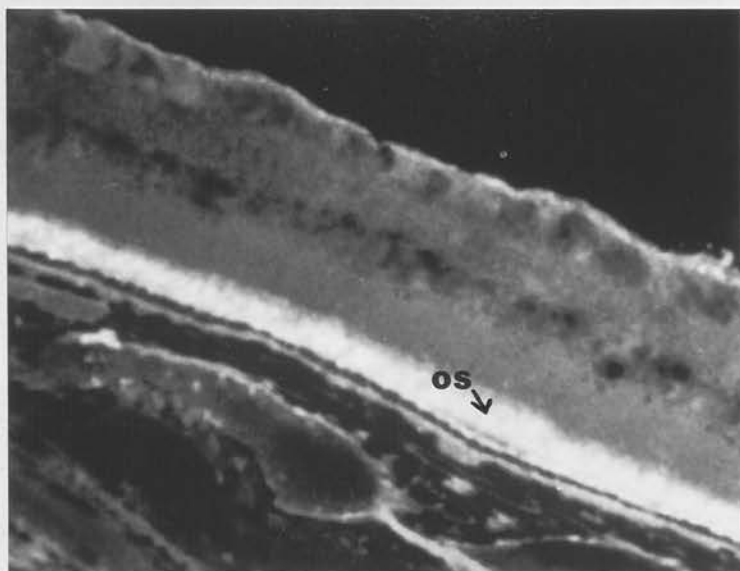
Two different cell lines secreting monoclonal antibodies were established. These were coded 1001.A1 and 1001.A3. The use of class-specific sheep anti-mouse γ globulin biotin conjugates in the staining procedure showed that both of these antibodies belong to the IgM class.

6.2.1. Analysis of the 1001.A1 antigen

6.2.1.1. Light microscopy

The 1001.A1 cell line was tested for the presence of retina-specific antibodies using fixed tissue sections of bovine neural retina. Immunoreactivity was detected by the immunofluorescence and peroxidase staining (2.2.2.) methods and was found to be specific to the

a



b

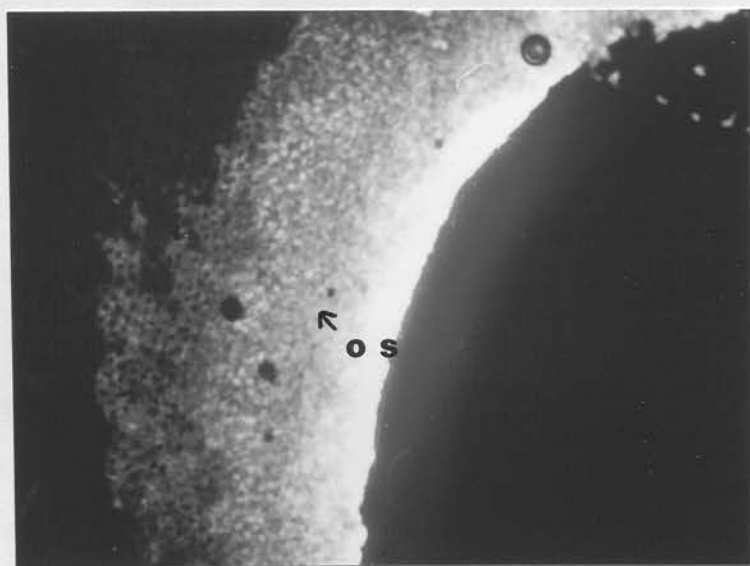


Figure 6.2.1. Photomicrographs of the bovine neural retina treated with 1001.A1 antibody followed by a biotinylated second antibody and Streptavidin-FITC as indicated in the text.

All photomicrographs were generated using a Biorad confocal laser microscope and image analyser.

a. Transverse tissue section of the bovine neural retina indicating staining in the outer segments (OS) of the photoreceptor cells and in the Interphotoreceptor Matrix (IPM). (Magnification X 150)

b. Oblique tissue section of the bovine neural retina indicating staining associated with the photoreceptor cells. (magnification X 600)

outer segments of the photoreceptors and to the IPM where there was intense staining of the tissue. Faint staining was also visualised in the photoreceptor cell bodies, indicating a lesser degree of immunoreactivity in this region, perhaps due to a lower concentration of the antigen (Figure 6.2.1.). Using a higher resolution to visualise 1001.A1 binding could not be obtained.

6.2.1.2. High resolution light microscopy

Higher magnifications of the stained tissue sections would give a higher resolution of the antibody localisation, and may distinguish whether the immunoreactivity is membrane-associated, cytosolic or specific to some cellular organelle. For this to be possible, it is necessary to produce much thinner sections of tissue (1 μ m), which requires embedding the tissue in hard plastic rather than in OCT. Higher resolution light microscopy and also electron microscopy may then be used.

1 μ m tissue sections were cut of the bovine neural retina and treated to visualise 1001.A1 binding using the colloidal gold-silver enhancement method (2.3.1.). No staining was observed. Sections were also treated to visualise lectin binding (ConA and PNA) using the colloidal gold-silver enhancement method (2.3.2.). In both cases the pattern and intensity of staining appeared similar to that seen on the 15 μ m sections. This confirmed that the colloidal gold-silver enhancement staining method was viable and that the lack

of antibody staining was for some other reason. It appeared likely either that the antigenicity of the tissue was lost during the embedding process or that the penetration of the antibody molecules into the tissue was hindered by the Lowicryl in which it was embedded. Higher magnifications providing a higher resolution to visualise 1001.A1 binding could not be obtained. Further characterisation of the 1001.A1 antigen was therefore continued by biochemical rather than histochemical methods.

6.2.1.3. Immunodotting of 1001.A1

Immunodotting was carried out (3.2.1.), using IPM as the antigen mixture, and 1001.A1 culture medium in the first incubation solution.

In the process of immunodotting, the antigen maintains its native state, minimising any losses in its antigenicity, and so maximising the probability of detecting antibody binding.

1001.A1 showed immunoreactivity with the IPM and so the antigen would appear to be present in the IPM (no reactivity was detected in control dots treated identically except that 1001.A1 was omitted from the first incubation).

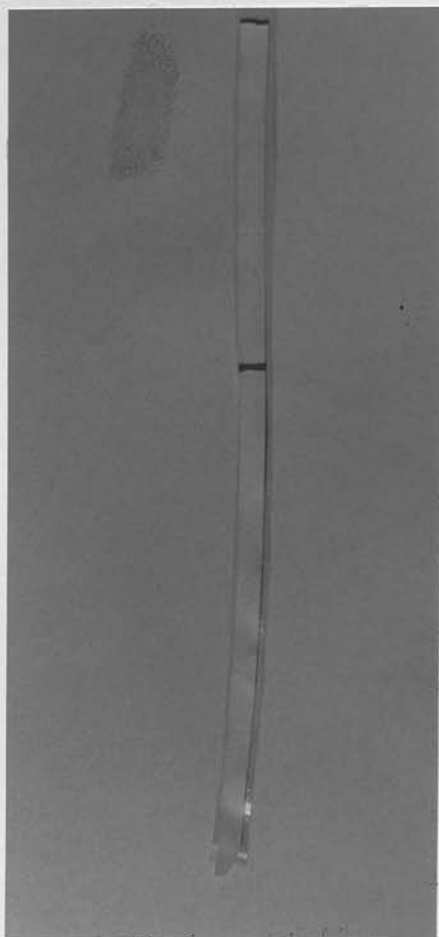


Figure 6.2.2. A strip cut from a Western blot of a 5-15% gradient polyacrylamide gel loaded with 500ug of IPM. The blot was treated with 1001.A1 followed by biotinylated second antibody, streptavidin-HRP and stained with chloronaphthol. See text for experimental detail.

6.2.1.4. Western blotting of 1001.A1

In order to identify the molecular weight of the 1001.A1 antigen, Western blotting was carried out.

IPM was subjected to SDS polyacrylamide gel electrophoresis (4.1.1.), the gel blotted (4.3.1.) and the nitrocellulose stained to visualise antibody binding (3.2.2.).

Immunoreactivity was seen as a single stained band on an area of the nitrocellulose, corresponding to a high molecular weight substance (Figure 6.2.2.).

Amido black staining of another strip of nitrocellulose from the same blot showed IRBP to be present on a similar area of the nitrocellulose to this antibody-binding component of the IPM.

Bovine IRBP has a molecular weight of 140 KDaltons in its monomeric state (Fong et al 1984a) and is a major component of the IPM (Adler and Kluckznik 1982).

Further experiments were carried out to find whether this high molecular weight material which binds to 1001.A1 was in fact IRBP.

Strips of nitrocellulose were cut from a Western blot of IPM and these were then treated with ConA, WGA (using the lectin-HRP method), (4.2.3.3.) or 1001.A1. The strips originated from the same Western blot and so it was possible to compare staining patterns between the different nitrocellulose strips. Those strips treated with Con A, WGA or 1001.A1, all stained in exactly the same area of the nitrocellulose (Figures 6.2.2.,6.2.3.),

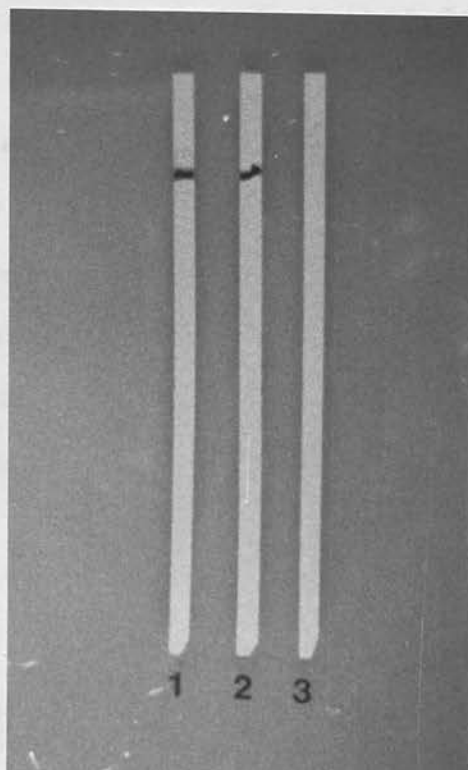


Figure 6.2.3. Strips cut from a Western blot of a 10% polyacrylamide gel loaded with 600ug of IPM. The blots were treated with lectins conjugated to HRP or with ConA followed by HRP, and stained with chloronaphthol. See text for experimental detail.

1. ConA.
2. WGA.
3. PNA.

indicating that the ConA, WGA and 1001.A1 all bind to components of the IPM with the same molecular weight. This area of the nitrocellulose remained unstained on the strip treated to visualise PNA-binding components of the IPM.

IRBP is known to bind ConA and WGA but not PNA (Fong et al 1984a), and so the fact that ConA, WGA and 1001.A1 all bind to this high molecular weight component of the IPM, while PNA does not, suggests that 1001.A1 does in fact bind IRBP.

To confirm this possibility, further dot blotting experiments were carried out this time, using a purified fraction of IRBP (1mg/ml) (5.2.) as the antigen. 1001.A1 immunoreactivity was confined to the IRBP purified fraction of the IPM (Table 6.2.1.).

Table 6.2.1. Dot blotting experiments using different antigen solutions and the monoclonal antibodies 1001.A1 and 1001.A3

Monoclonal antibody (Mab)	Antigen solution		
	IPM	IRBP	IPM-IRBP
no Mab	(-)	(-)	(-)
1001.A1	(+)	(+)	(-)
1001.A3	(+)	(-)	(+)

(-) no immunoreactivity

(+) immunoreactivity

IPM-IRBP IPM depleted of IRBP

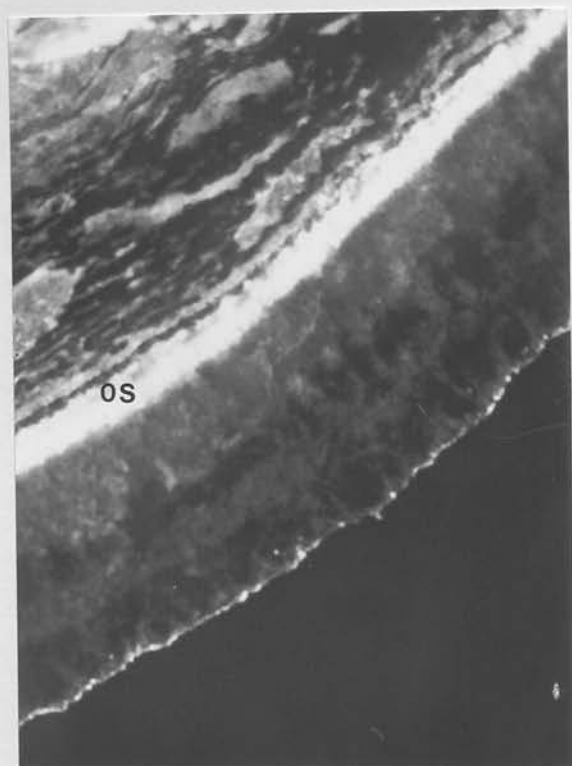
6.2.1.5. 1001.A1 binding to other tissue types

The monoclonal antibody was tested for immunoreactivity on fixed sections of other tissue types by Mr A Stewart, (an Honours student of the Department of Biochemistry), using the immunofluorescence method. No fluorescence was visualised on fixed tissue sections of bovine brain stem, cerebellum, hypothalamus or adrenal medulla and cortex, in agreement with the lack of IRBP in these tissues.

6.2.1.6. Summary of the 1001.A1 antigen analysis

In conclusion, 1001.A1 shows immunoreactivity with the IRBP, present only in the IPM and associated with the photoreceptor cells.

a



b

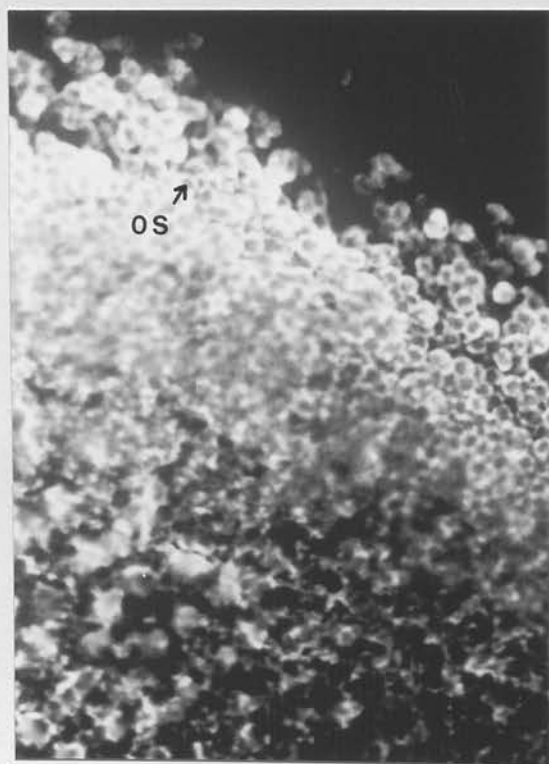


Figure 6.2.4. Photomicrographs of the bovine neural retina treated with 1001.A3 antibody followed by a biotinylated second antibody and Streptavidin-FITC as indicated in the text.

- a. Transverse tissue section of the bovine neural retina indicating staining in the outer segments (OS) of the photoreceptor cells and in the Interphotoreceptor Matrix (IPM). (magnification: X 150)
- b. Oblique tissue section of the bovine neural retina indicating staining around the photoreceptor cells. (magnification: X 900)

6.2.2. Analysis of the 1001.A3 antigen

6.2.2.1. Light microscopy

The distribution of 1001.A3 binding was visualised on fixed tissue sections of bovine neural retina by the immunofluorescence method. Immunoreactivity was localised to the outer segments of the photoreceptor cells and to the IPM (Figure 6.2.4.). No staining was apparent in the cell bodies of the photoreceptor cells. When tissue sections cut tangentially with respect to the photoreceptor cells were studied (Figure 6.2.4.), immunoreactivity was seen to be restricted to an area of the IPM surrounding the rod photoreceptor cells. The antigen appeared to form a distinct sheath-like structure around both the inner and outer segments of the photoreceptors. Binding of 1001.A3 was different to that of 1001.A1 which appeared to bind within a much less rigidly defined area. Although both antibodies show immunoreactivity within the same region of the retina, the localisation of their binding would appear to differ slightly.

It was not possible to determine the presence of 1001.A3 immunoreactivity within the photoreceptor cells, or within the cells of the pigmented epithelium at this resolution of microscopy. Unfortunately, as with 1001.A1, the antigenicity of the tissue was lost when it was embedded in Lowicryl K4M and so higher magnifications could not be used to further study the

binding pattern of 1001.A3. Biochemical techniques were therefore used to characterise and distinguish the 1001.A3 and 1001.A1 antigens.

6.2.2.2. Immunodotting of 1001.A3

Immunodotting was carried out (3.2.1.) using IPM (1mg/ml protein) as the antigen solution and a first incubation solution containing 1001.A3 culture medium. Very strong immunoreactivity was found in the IPM indicating that 1001.A3 binds to a component present in the IPM. This was repeated using either purified IRBP, or IPM in which the IRBP had been removed. 1001.A3 was seen to bind strongly to the fraction of IPM from which IRBP had been removed and not with the purified IRBP fraction (Table 6.2.1).

This confirmed that 1001.A3 shows a different binding specificity to that of 1001.A1, and does not bind to IRBP, but to some other component of the IPM.

6.2.2.3. Western blotting of 1001.A3

Western blotting was then carried out to identify this component of the IPM. A 6-15% exponential acrylamide gradient SDS polyacrylamide gel was used. This was made by using a multi-channel pump to transfer, at equal flow rates (approximately 2ml/min) gel mixture containing 6% acrylamide into a 15% acrylamide gel mixture, and the mixed acrylamide solution into the gel cassette. The stacking gel consisted of a 5% acrylamide solution. The

nitrocellulose was treated to visualise 1001.A3 binding to IPM. Very faint chloronaphthol staining occurred on an area of the nitrocellulose corresponding to the stacking gel and to the top of the separating gel (the staining was too weak to be photographed and is not shown). This indicated that the antigen has a very high molecular weight, that it fails to penetrate the separating gel and that it is present in small amounts in the stacking gel or does not transfer efficiently to the nitrocellulose in Western blotting. A polyacrylamide gel loaded with IPM and stained for protein by the silver stain method (4.2.2.) showed this area of the gel to be apparently devoid of protein. Western blotting of 1001.A3 was repeated using a 2-dimensional gel system (4.1.2.). 70 μ l of IPM was loaded onto the 1st dimension IEF gel and the 2nd dimension consisted of a 6-15% acrylamide gradient SDS-polyacrylamide gel with a 5% acrylamide stacking gel. Following Western blotting, the nitrocellulose was treated to visualise 1001.A3 binding. Very weak staining occurred on an area of the nitrocellulose corresponding to the top of the SDS-polyacrylamide gel and near the basic end of the IEF gel (Figure 6.2.5.). A pH gradient was not fully established across the first dimension of this gel, probably due to too short a time being allowed for its electrophoresis. For this reason, little confidence can be given to the pI value obtained for the 1001.A3 antigen although its position at the top

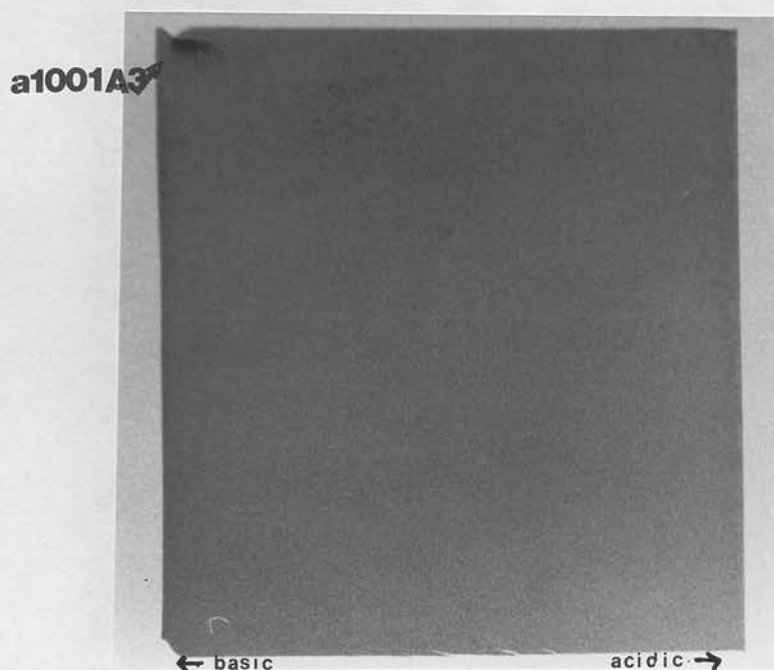


Figure 6.2.5. Chloronaphthol staining of a Western blot of a two-dimensional polyacrylamide gel loaded with 70ug of IPM. The blot was treated with 1001.A3 followed by biotinylated second antibody and Streptavidin-HRP. See text for experimental detail.

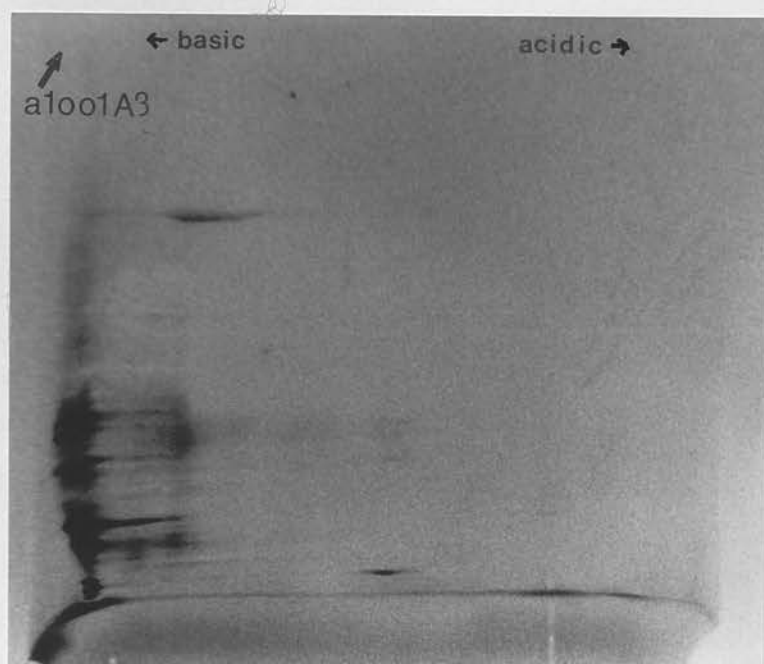


Figure 6.2.6. A two-dimensional polyacrylamide gel loaded with 70ug of IPM, silver stained for protein. See text for experimental detail.

of the gel suggests that it may be basic. However this method provided a means of concentrating the antigen in such a way as to allow it to be visualised by antibody treatment. The very high molecular weight of the antigen means it may have been impeded in the 1st dimension gel and may have been unable to move away from the basic end of the gel towards the more acidic end. Another gel treated identically and stained for protein by the silver stain method again showed the area associated with the antigen to be apparently devoid of protein (Figure 6.2.6.).

In conclusion the antigen is only detected on the basis of its immunoreactivity. It is not detected by protein staining, either because it stains poorly using conventional protein staining techniques, or because it is present in too low a concentration to be detected by these techniques. The very strong immunoreactivity (on the basis of dot blotting) would argue against the latter and would suggest that this high molecular weight antigen does in fact stain poorly and/or has difficulty in entering the SDS polyacrylamide gel systems used. It may also blot poorly, perhaps having difficulty in penetrating out from the polyacrylamide gel onto the nitrocellulose. This would lead to a reduction in the amount of antigen available for detection by the methods used above.

6.2.2.4. Immune precipitations of antigens using monoclonal antibodies

Attempts were made to immune precipitate the 1001.A3 and 1001.A1 antigens from IPM.

If successful this might allow the isolation of enough 1001.A3 antigen to make it possible to detect it by conventional staining methods and provide a method of purifying the antigen from the IPM for further biochemical analysis.

An enzyme-linked immunosorbent assay (ELISA) system was used to establish the conditions which would allow a fixed volume of monoclonal antibody solution to bind a maximum possible amount of antigen.

6.2.2.4.1. Enzyme-linked immunosorbent assay (ELISA)

This method is described in Section 3.2.3. Individual wells of 96-well microtitre plates were each coated with different amounts of IPM (0.5 ug-10 ug of protein/well) and the plates were incubated with first antibody solution containing 1001.A1, 1001.A3 culture medium, 223.C3 ascitic fluid (6.2.2.4.11.) or antibody-diluting solution.

In the case of 1001.A1 culture medium a maximal absorbance value was obtained with those wells coated with 2 ug of protein (the equivalent of 2 ul of IPM) when incubated with 100 ul of 1001.A1 culture medium.

Table 6.2.2. The ratio of monoclonal antibody to antigen (v/v), which allows maximal binding of antigen to a set volume of monoclonal antibody.

monoclonal antibody (Mab)	ratio of Mab:IPM (v/v)
1001.A1	50:1
1001.A3	20:1
223.C3 (Asc)	1:2

(Asc) = Ascitic fluid

This indicated a ratio of 50:1 1001.A1 culture medium:IPM (v/v) is necessary for maximal antigen binding to monoclonal antibody (Figure 6.2.7a.). Wells were then coated with 2 ug of protein and incubated with different volumes of 223.C3 ascitic fluid. Figure 6.2.7b. shows the absorbance values obtained and indicates that monoclonal antibody binding is not saturated with this volume of antigen and so for this monoclonal antibody the values obtained from Figure 6.2.7a. are valid. The equivalent absorbance values are not shown for 1001.A1 or 1001.A3, but it was obvious that the same was also true for these, as the absorbance values increased when larger volumes of monoclonal antibody solution were incubated in wells coated with equal amounts of antigen. The ratios for optimal binding of the other monoclonal antibodies used are shown in Table 6.2.2.

These ratios of antigen to antibody were used in the incubation steps of immune precipitations.

It seemed sensible initially to try the immune-precipitation of IRBP from IPM using 1001.A1, since IRBP can be easily detected and recognised. This would allow the technique to be perfected before being adapted to the immune precipitation of a molecule of unknown properties such as the 1001.A3 antigen in the IPM.

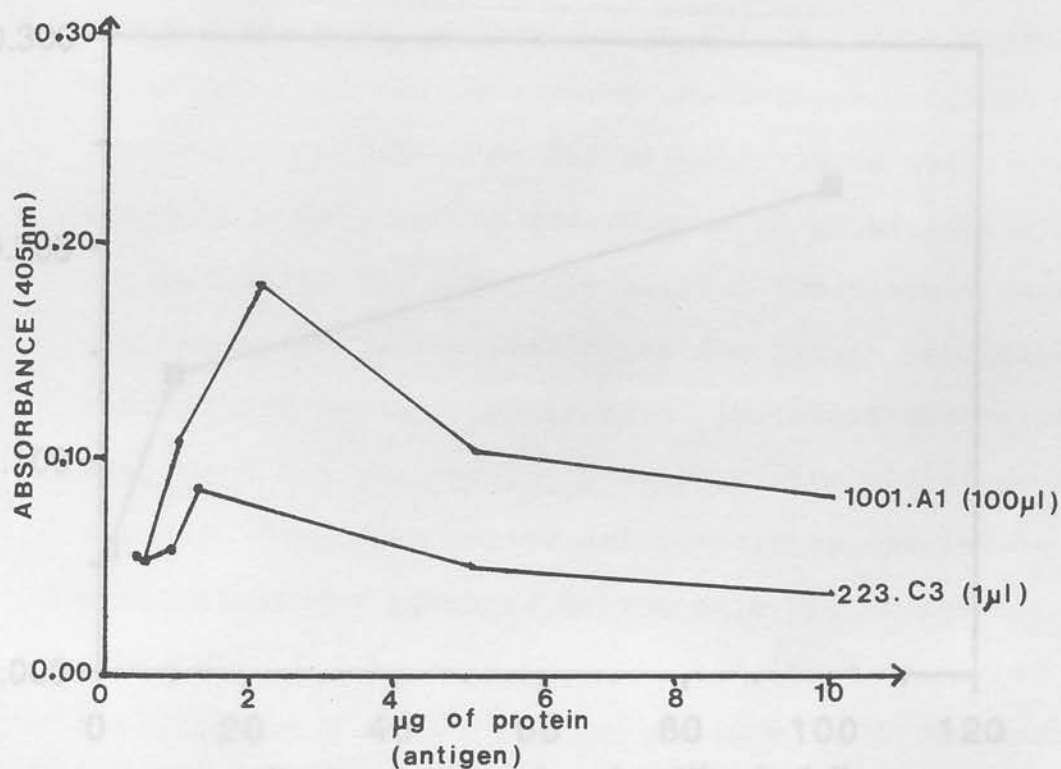


Figure 6.2.7.a The absorbance values from ELISA plates coated with varying amounts of antigen and treated with monoclonal antibody. See text for experimental detail.

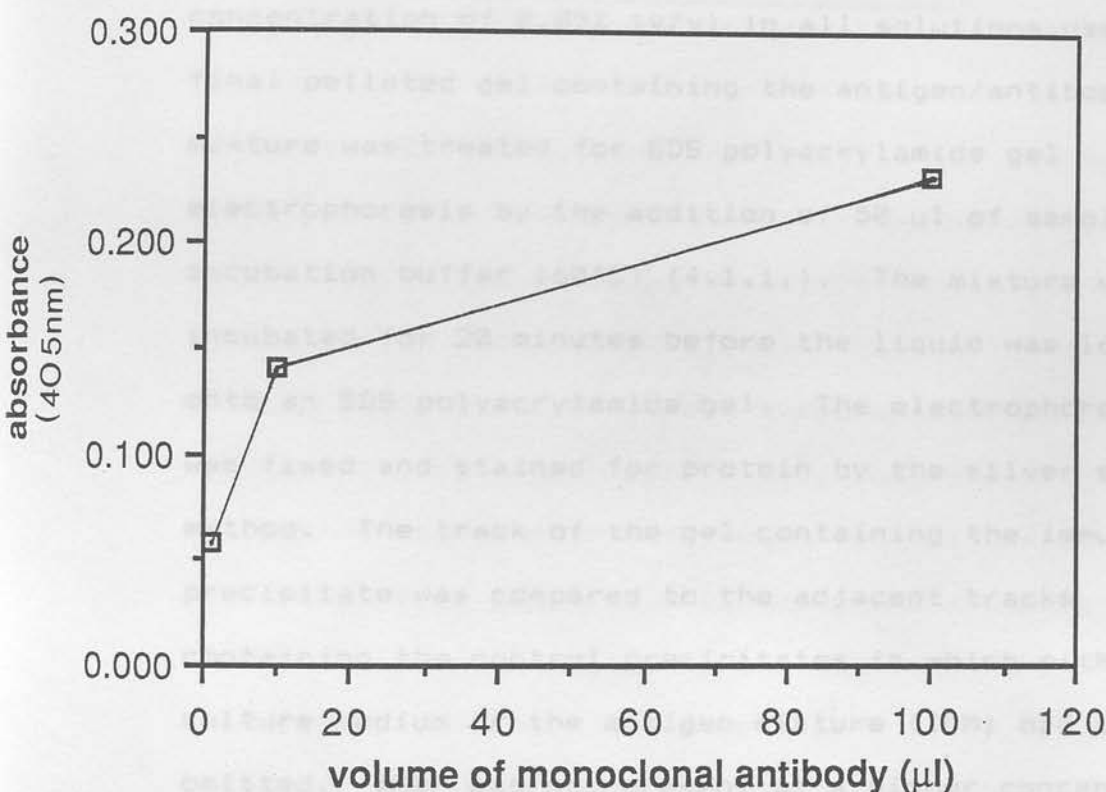


Figure 6.2.7.b The absorbance values from ELISA plates coated with 2 μg of protein and treated with varying amounts of 223.C3 ascitic fluid. See text for experimental detail.

6.2.2.4.2. Immune precipitation of IRBP from IPM using 1001.A1

This was carried out essentially by the method described for the immune precipitation of horseradish peroxidase (3.2.4.1.) except that 1.5ml of 1001.A1 culture medium was incubated with 1ml of sheep anti-mouse γ globulin-Sepharose 4B conjugate, 30 μ l of IPM was added as the antigen solution and Tween 20 was added to a final concentration of 0.05% (v/v) in all solutions used. The final pelleted gel containing the antigen/antibody mixture was treated for SDS polyacrylamide gel electrophoresis by the addition of 50 μ l of sample incubation buffer (60°C) (4.1.1.). The mixture was incubated for 20 minutes before the liquid was loaded onto an SDS polyacrylamide gel. The electrophoresed gel was fixed and stained for protein by the silver stain method. The track of the gel containing the immune precipitate was compared to the adjacent tracks containing the control precipitates in which either the culture medium or the antigen mixture (IPM) had been omitted. IRBP was not present at a higher concentration in the experimental fraction than in the control fractions and so appeared not to have been precipitated by this procedure.

It was not clear why this procedure did not produce a successful precipitation. One mg of sheep anti-mouse γ globulin was available on the Sepharose 4B gel for binding the monoclonal antibody present in the culture

medium (estimated to contain 1.5 ug-15 ug of monoclonal antibody in 1.5ml of culture medium) and enough antigen was used to be detected after SDS polyacrylamide gel electrophoresis even allowing for losses of up to 60% (leaving 12 ug of protein, easily detected by the silver staining method). One possible explanation was that the monoclonal antibody was unable to bind to the solid state second antibody due to the limited number of sheep anti-mouse IgM antibodies present. The method used to prepare the γ globulin fraction for coupling to the Sepharose 4B involves the precipitation of a sheep anti-mouse γ globulin fraction. Normal mouse serum contains on average 1-10mg/ml of γ globulin, the majority being IgG molecules and only 5% of which may be IgM molecules (Goding 1983) and therefore a sheep anti-mouse γ globulin fraction may contain predominantly anti-mouse IgG antibodies rather than anti-mouse IgM antibodies. The availability of anti-IgM antibodies for coupling to Sepharose 4B would therefore be much lower than that of anti-IgG antibodies. For this reason it is likely that the majority of the antibody molecules coupled to the Sepharose 4B are of the anti-mouse IgG type rather than anti-mouse IgM molecules. ~~is stated by the suppliers to~~ An insufficient number of second antibody molecules with the ability to bind 1001.A1, an IgM class antibody would lead to a reduction in the amount of IRBP which could be precipitated. The same sheep anti-mouse γ globulin fraction is used to prepare the sheep anti-

mouse γ globulin-biotin conjugate, used in the initial screening of the monoclonal antibodies by the immunofluorescence method. This fraction does therefore contain some anti-mouse IgM molecules. The immunofluorescence method does, however, deal with very small quantities of antigen and antibody (not easily quantified) presumably at low enough levels for the anti-IgM molecules present in the sheep anti-mouse globulin-biotin conjugate solution to be in sufficient quantity to bind to the small concentration of monoclonal antibody bound to the tissue section. To increase the number of anti-mouse IgM antibody molecules available for binding the 1001.A1 IgM monoclonal antibody, the solid state γ globulin was replaced by commercial goat anti-mouse IgM (μ chain-specific) antibody conjugated to agarose beads.

6.2.2.4.3. Immune precipitation of IRBP using solid state goat anti-mouse IgM antibodies

The above process was repeated except that the solid state anti-mouse γ globulin was replaced by 50 μ l of the goat anti-mouse IgM molecules conjugated to agarose beads. This volume of gel is stated by the suppliers to be capable of binding 20 μ g of mouse IgM antibodies, more than is present in 1.5ml of culture medium (assuming 1-10 μ g/ml of IgM/ml of culture medium).

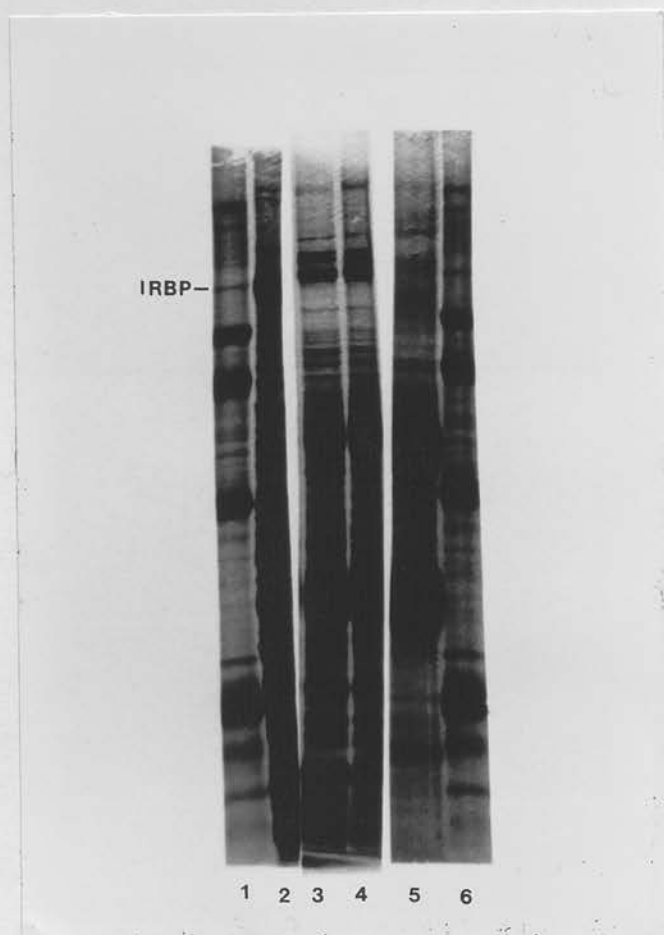


Figure 6.2.8. Attempted immune precipitations of IRBP from IPM using 1001.A1, visualised on a 10% polyacrylamide gel, silver stained for protein. See text for experimental detail.

1. molecular weight markers.
2. IPM (80ug).
3. aIgM + IPM (no 1001.A1) (70ul fraction).
4. aIgM + 1001.A1 + IPM (70ul fraction).
5. aIgM + 1001.A1 (noIPM) (70ul fraction).
6. molecular weight markers.

Again the silver stained SDS polyacrylamide gels of the presumptive immune precipitate failed to show any immune-precipitated IRBP (Figure 6.2.8.).

It seemed, that the detection methods being used to visualise the precipitated antigen were too insensitive and the background levels of non-specific binding too high. One method of raising the sensitivity levels is to label the antigen in such a way that lower concentrations of the antigen may be detected by means of its label, whereas unlabelled molecules would not be detected.

Two labelling methods were used; the proteins in the IPM were covalently labelled with either ^{125}I -iodine or with NHS-biotin.

6.2.2.4.4. ^{125}I -labelling of IPM proteins

The proteins in the IPM were labelled with ^{125}I -iodine by the Chloramine T method (4.6.1.). A 10 μl sample of the labelled IPM was taken for radioactive counting.

This contained 2.56×10^6 counts per minute (cpm). With an efficiency of counting ^{125}I of 80% this corresponds to approximately 3×10^6 disintegrations per minute (dpm), that is, 5×10^4 disintegrations per second (dps). Thus 10 μl of ^{125}I -IPM contains approximately $5 \times 10^4 / 37 \times 10^6 \text{ mCi}$, that is, 1.3 μCi (since $1 \text{ mCi} = 37 \times 10^6$ Bequerel (Bq) = $37 \times 10^6 \text{ dps}$).

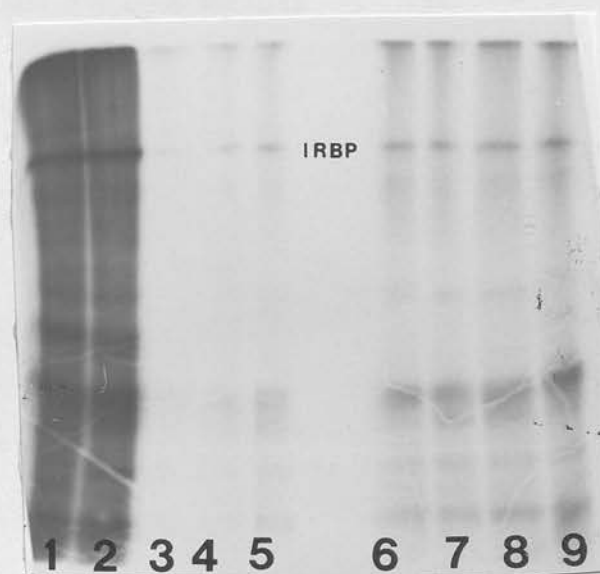


Figure 6.2.9. Autoradiograph of the immune precipitation of ^{125}I -labelled antigen by 1001.A1 or 1001.A3 using anti-mouse IgM-agarose (aM-IgM) (samples in a final volume of 50ul). See text for experimental detail.

1. ^{125}I -IPM (10ul).
2. ^{125}I -IPM (5ul).
3. 1001.A1 + 25ul aM-IgM + ^{125}I -IPM.
4. 1001.A1 + 50ul aM-IgM + ^{125}I -IPM.
5. 1001.A1 + 75ul aM-IgM + ^{125}I -IPM.
6. 1001.A3 + 25ul aM-IgM + ^{125}I -IPM.
7. 1001.A3 + 50ul aM-IgM + ^{125}I -IPM.
8. 1001.A3 + 75ul aM-IgM + ^{125}I -IPM.
9. 50ul aM-IgM + ^{125}I -IPM.

To check that ^{125}I label was present on IRBP, 10 μl of the treated IPM was subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. This showed that IRBP did appear to be labelled (Figure 6.2.9.).

6.2.2.4.5. Immune precipitation of IRBP from ^{125}I -labelled IPM

The immune precipitation of ^{125}I -IRBP from the ^{125}I -IPM was carried out as described in Section 6.2.2.4.2., except that 10 μl of IPM containing approximately 1.3 μCi of ^{125}I -iodine covalently bound to protein (6.2.2.4.4.) was added to the IPM before being used. The immune precipitate was treated by SDS polyacrylamide gel electrophoresis and X-ray film was exposed to the radioactive gel overnight before being developed. The IRBP was detected at equal levels in the controls and in the immune precipitates (Figure 6.2.9.). indicating that either no IRBP was precipitated or that the 1001.A1 monoclonal antibody is selectively binding the unlabelled IRBP which cannot be detected by this method. It is possible that this method of labelling the IRBP may alter the structure of the antigenic determinant so that it is no longer recognised by the antibody.

6.2.2.4.6. NHS-Biotin labelling of IPM proteins

NHS-biotin was used to label the proteins in IPM by the method described in Section 4.6.3.1. Labelling was confirmed by Western blotting an SDS polyacrylamide gel

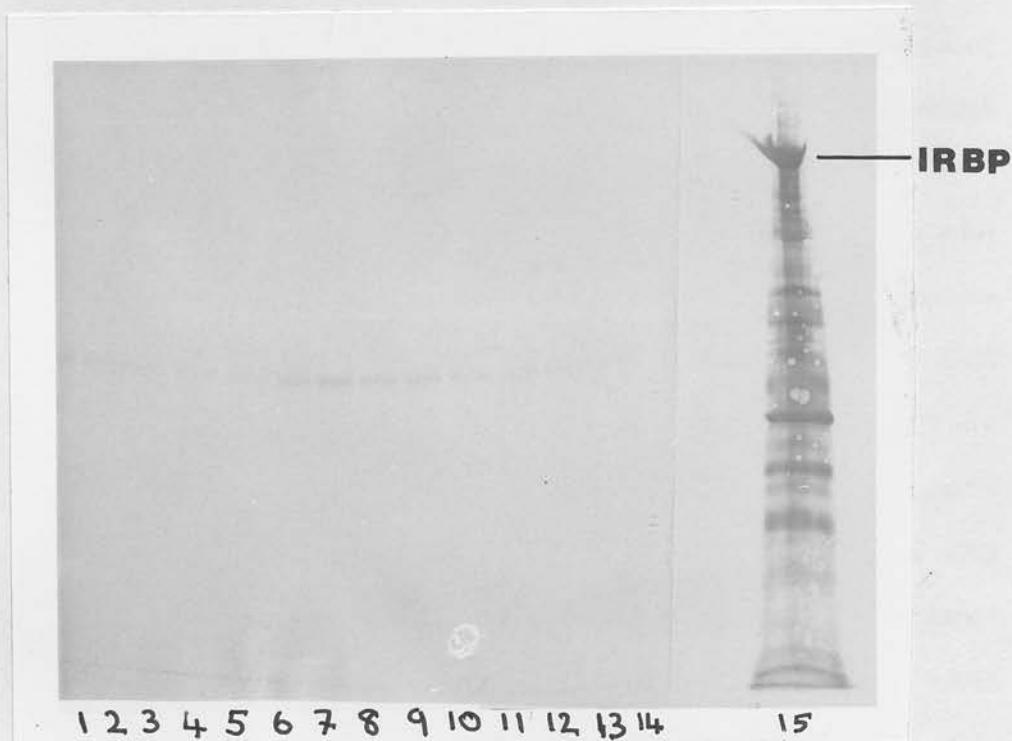


Figure 6.2.10. Western blot of the immune precipitation of a biotinylated antigen by 1001.A1 using anti-mouse IgM-agarose (aM-IgM). Samples had a final volume of 50ul, see text for experimental detail.

1. 20ul IPM-bio + 500ul 1001.A1 + 20ul aM-IgM.
2. 40ul IPM-bio + 500ul 1001.A1 + 20ul aM-IgM.
3. 20ul IPM-bio + 500ul 1001.A1 + 40ul aM-IgM.
4. 40ul IPM-bio + 500ul 1001.A1 + 40ul aM-IgM.
5. 20ul IPM-bio + 2.5ml 1001.A1 + 20ul aM-IgM.
6. 40ul IPM-bio + 2.5ml 1001.A1 + 20ul aM-IgM.
7. 20ul IPM-bio + 2.5ml 1001.A1 + 40ul aM-IgM.
8. 40ul IPM-bio + 2.5ml 1001.A1 + 40ul aM-IgM.
9. 20ul IPM-bio + 5.0ml 1001.A1 + 20ul aM-IgM.
10. 40ul IPM-bio + 5.0ml 1001.A1 + 20ul aM-IgM.
11. 20ul IPM-bio + 5.0ml 1001.A1 + 40ul aM-IgM.
12. 40ul IPM-bio + 5.0ml 1001.A1 + 40ul aM-IgM.
13. 20ul IPM-bio + 40ul aM-IgM.
14. 40ul IPM-bio + 40ul aM-IgM.
15. IPM-bio (70ul).

containing the biotinylated fraction, followed by staining the nitrocellulose to visualise the biotinylated material as for treatment with lectin. IRBP did appear to be labelled (Figure 6.2.10.).

6.2.2.4.7. Immune precipitation of IRBP from biotinylated-IPM

The immune precipitation was carried out essentially as before, replacing the IPM with biotinylated-IPM. As it was not certain that the biotinylated IRBP would bind to 1001.A1 to the same extent as does unlabelled IRBP, various ratios of IRBP:1001.A1 were used in the immune precipitations. The immune precipitate was subjected to SDS polyacrylamide gel electrophoresis as above and then to Western blotting. The nitrocellulose was treated to visualise the presence of biotinylated-protein (6.2.2.4.6.). Chloronaphthol staining of the nitrocellulose occurred as a single band (Figure 6.2.10.). This was present in all the precipitation fractions, including those in which either the monoclonal antibody or the second antibody had been omitted. This protein was therefore not specifically immune precipitated, and the staining of the nitrocellulose indicated some non-specific binding occurring, probably in the staining procedure between the streptavidin-HRP conjugate and the antibody molecules in the precipitation mixture.

6.2.2.4.8. Other anti-IRBP monoclonal antibodies

The monoclonal antibodies 1001.B4, 1001.C4 and 223.C3 (raised in a similar manner to 1001.A1 as described in Section 6.2.) have been shown to react with IRBP. They have the ability to bind to purified IRBP (seen by immunodotting experiments using purified IRBP as the antigen solution). Epitope analysis has not yet been carried out, so these monoclonal antibodies may or may not share common antigenic determinants on the IRBP molecules.

6.2.2.4.9. Immune precipitations using other anti-IRBP monoclonal antibodies

Attempts were made to immune precipitate IRBP from the IPM using each of the monoclonal antibodies 1001.B4, 1001.C4 and 223.C3. IPM and biotinylated-IPM were used as described above.

None of these monoclonal antibodies immune precipitated the IRBP.

The availability of several different monoclonal antibodies directed towards the same molecule led to the possibility of producing a synthetic "multi-clonal" antibody against the IRBP (assuming the antigenic determinant for each monoclonal antibody is not the same). It was thought that such a "multi-clonal" antibody would bind to antigen molecules more tightly than single monoclonal antibodies, increasing the possibility of cross-linking occurring between the

antigen and different antibody molecules. For this reason, immune precipitations were attempted using such a "multi-clonal" antibody.

6.2.2.4.10. Immune precipitation of IRBP from IPM using "multi-clonal" antibodies

The immune precipitation of IRBP from IPM was attempted using a synthetic "multi-clonal" antibody solution containing equal volumes of each anti-IRBP monoclonal antibody culture medium available, in a total volume of 1.5ml (to replace the 1.5ml of 1001.A1 culture medium used previously).

Again, the IRBP was not immune precipitated.

There was still the possibility that not enough monoclonal antibody was binding to the solid state second antibody to allow enough antigen to bind and to be detected. To increase the concentration of monoclonal antibody in solution available for binding both the solid state second antibody and antigen, ascitic fluid was used.

6.2.2.4.11. 223.C3 Ascitic fluid

Ascitic fluid containing 223.C3 monoclonal antibody was produced (3.3.1.) and partially purified by DEAE-cellulose ion exchange chromatography (3.3.2.).

When used in the ELISA system, it was found that 223.C3 ascitic fluid could bind much more IRBP than could an equal volume of 1001.A1 culture media (Table 6.2.2.).

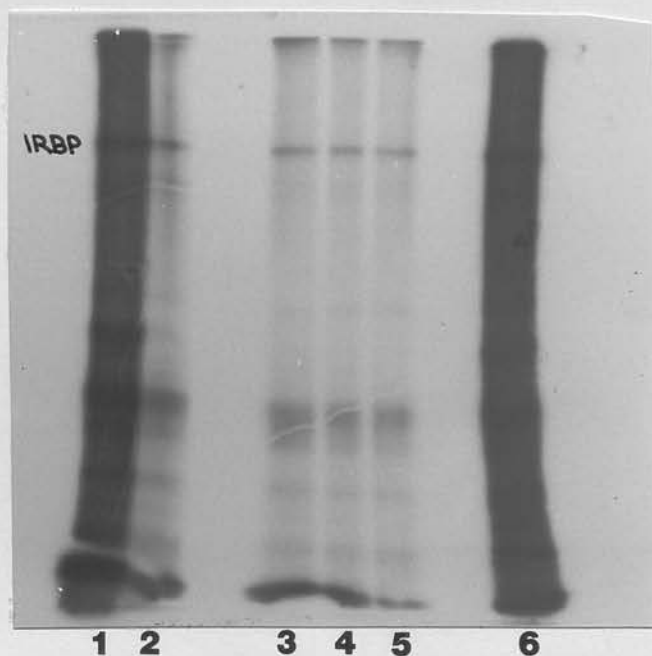


Figure 6.2.11. Autoradiograph of the immune precipitation of ^{125}I -labelled antigen by 223.C3 ascites using anti-mouse IgM-agarose (aM-IgM). Samples in a final volume of 50ul, see text for experimental detail.

1. ^{125}I -IPM (10ul).
2. 50ul aM-IgM + ^{125}I -IPM
3. 75ul aM-IgM + 223.C3 ascites + ^{125}I -IPM.
4. 50ul aM-IgM + 223.C3 ascites + ^{125}I -IPM.
5. 25ul aM-IgM + 223.C3 ascites + ^{125}I -IPM.
6. ^{125}I -IPM (10ul).

6.2.2.4.12. Immune precipitation of IRBP from IPM using 223.C3 ascitic fluid

Attempts were made to immune precipitate IRBP from the IPM using 223.C3 ascitic fluid to replace the 1001.A1 culture medium used previously. A 50 ul aliquot of purified 223.C3 ascitic fluid was used to replace the 1001.A1 culture medium and 100 ul of IPM (or biotinylated-IPM), was used instead of 30 ul of IPM. This achieved optimal conditions for the antibody:antigen binding as found by the ELISA method (6.2.2.4.1.) (Table 6.2.2.).

Again the monoclonal antibody apparently failed to immune precipitate the IRBP (Figure 6.2.11.).

It was unlikely in this case that there was too little monoclonal antibody present to allow enough IRBP to be immune precipitated and detected.

The increased sensitivity gained by the use of biotinylated-IPM may counteract its usefulness by reducing the binding capacity of the monoclonal antibody to the antigen. It is possible that the biotinylation of the antigen may alter its structure so that it is no longer recognised or is poorly recognised by the antibody molecules.

Another reason for the lack of success in this immune precipitation method would be explained by the inability of the monoclonal antibody to bind to both the solid state second antibody and to the antigen at the same time, perhaps due to steric hindrance.

6.2.2.4.13. Immune precipitation of the 1001.A3 antigen from the IPM using 1001.A3

A final attempt using IPM, ^{125}I -IPM or biotinylated-IPM with 1001.A3 culture medium, to immune precipitate the 1001.A3 antigen was carried out.

The same conditions as for 1001.A1 were used except that 1.5 ml of 1001.A1 culture medium was replaced by 1.5ml of 1001.A3 culture medium; and either 75 μl of IPM, 75 μl of biotinylated IPM, or 75 μl of IPM containing 10 μl of ^{125}I -IPM (containing 1.3 μCi of ^{125}I) was used, according to the ratio of antibody to antigen required for optimal binding, as determined by the ELISA method (Table 6.2.2.). The presumptive immune precipitate was subjected to SDS-polyacrylamide gel electrophoresis, Western blotting or autoradiography, depending on the label. Silver stained gels failed to visualise any precipitate, stained nitrocellulose failed to visualise a biotinylated-precipitate and the autoradiographs failed to visualise a radio-labelled precipitate (Figure 6.2.9.).

It is possible in this case that it is not the immune precipitation procedure but the ability of the antigen to enter SDS-polyacrylamide gel systems that is the limiting factor in detecting precipitated antigen. Tests were carried out to find which step in the immune precipitations may have been responsible for the failures. A 50 μl aliquot of solid state anti-mouse IgM-agarose conjugate was mixed with 1.5ml of 1001.A3

culture medium, incubated for 2 hours and then spin washed as before. To the pelleted gel was added 1.5ml of a 1000-fold dilution of a stock sheep anti-mouse-HRP conjugate solution in TBS/BSA(1mg/ml), and this was incubated for a further 2 hours. The gel was spin washed again before the addition of 4-chloro-1-naphthol stain (2.2.2.) to visualise the presence of HRP. The pelleted gel became purple whereas the control gel treated identically (except that the 1001.A3 monoclonal antibody was omitted) remained white. This indicated that the 1001.A3 monoclonal antibody had bound to the gel and that the sheep anti-mouse-HRP conjugate had in turn, bound to the 1001.A3 antibody. This suggests that the failure to immune precipitate the 1001.A3 antigen was due either to a failure in antigen:monoclonal antibody binding, or in the detection system used to visualise the precipitated antigen.

6.2.2.4.14. Summary of the immune precipitation experiments

It is unclear as to why these immune precipitations were not successful. There may be different reasons for the failures using the different antibodies.

It is possible that in all cases the labelled antigen is no longer recognised by the monoclonal antibody or that the monoclonal antibody favours binding to the unlabelled antigen. This is unlikely to have occurred

for every antibody used as the ^{125}I label is directed towards tyrosine residues whereas the biotin is towards the amino groups of lysine residues.

A second possibility is that the monoclonal antibodies are incapable of binding to both the solid state second antibody and to the antigen at the same time. This may be sterically unfavourable due to the large size of the IgM antibody molecules which, when in close proximity to the gel or to each other, may hinder antigen binding (especially if the antigen molecule is also large).

The monoclonal antibodies may have low affinities for their antigen and may not be able to precipitate the antigen. This is especially true for the 1001.A1 antibody. IRBP is the major glycoprotein constituent of IPM whereas proteoglycans and glycosaminoglycans are said to comprise only 2% (w/w) of the IPM (1.8.) and yet the intensity of chloronaphthol staining in dot blotting experiments indicates a lesser degree of binding of 1001.A1 to IRBP than of 1001.A3 to the 1001.A3 antigen.

The difference in binding capacities of the two antibodies may be due to the presence of a higher number of antibody binding sites per individual molecule in the case of the 1001.A3 antigen as compared to IRBP.

Proteoglycan molecules are composed of identical repeated units of carbohydrate and so an antigenic determinant may be present many times in a single proteoglycan molecule. In comparison, it is possible that the peptide structure of the IRBP molecule which is

recognised by 1001.A1 is present only once in each IRBP sub-unit. It seems likely that the affinity of 1001.A1 to IRBP is the limiting factor in the immune precipitation procedures and that the antibody is incapable of immune precipitating the IRBP.

A third possibility (in the case of 1001.A3) is that the immune precipitation procedure does in fact work but the detection methods are not suitable for the antigen. It would appear that the use of immune precipitation procedures as a method of isolating the 1001.A3 antigen in order to characterise it biochemically is not feasible and that some other method is required for its isolation. The inappropriateness of these detection methods became apparent when further analyses of the 1001.A3 antigen molecule was carried out (6.2.2.15).

The next step taken was to make use of standard biochemical protein separation methods to fractionate the IPM with the purpose of testing each fraction for its ability to bind the monoclonal antibody. The components of the fractions with the ability to bind the monoclonal antibody would then be characterised biochemically.

The IPM was initially fractionated according to molecular weight by gel filtration (size exclusion) chromatography.

6.2.2.5. Gel filtration chromatography of IPM

500 μ l aliquots of IPM (1mg/ml of protein) was subjected to gel filtration chromatography using a Superose 12 10/30 HR FPLC column (4.8.4.)(Figure 6.2.12). Thirty-two 1ml fractions were collected and the constituents of each tested by immunodotting for their ability to bind 1001.A3, (3.2.1.), using the fractions collected as the antigen solutions (Table 6.2.3.).

Three successive fractions were found to contain components to which 1001.A3 could bind, the second of the three containing the highest concentration of these (corresponding to the immunodot which was the most highly stained with chloronaphthol). The retention time of this fraction indicated that the material collected had been excluded from the pores of the gel. According to the suppliers, the Superose 12 10/30 HR gel has an exclusion limit of 2×10^6 Daltons for globular proteins, indicating that the material to which 1001.A3 binds has a molecular weight, or is part of a mixture of components with an overall molecular weight of greater than 2×10^6 Daltons. The fraction which stained the most intensely in the dot blotting experiments was subjected to SDS polyacrylamide gel electrophoresis and the gel was silver stained for protein or stained for carbohydrate by the PAS method (4.2.3.). No protein or carbohydrate was visualised after staining. The very high molecular weight of the substance led to the suggestion that it may not be able to enter a 10% SDS

polyacrylamide gel and therefore a 5% SDS polyacrylamide gel was used and again silver staining of the gel failed to show any protein present. The process was repeated using a 3% SDS polyacrylamide gel but due to the flimsy nature of such a low percentage acrylamide gel, it proved impossible to carry out the silver staining procedure. Instead, the less sensitive but simpler Coomassie blue staining method was used. No protein was visualised after staining, indicating that either there was no protein present, that it was present in too low a concentration to be detected by the Coomassie blue stain, or that it failed to stain by this method.

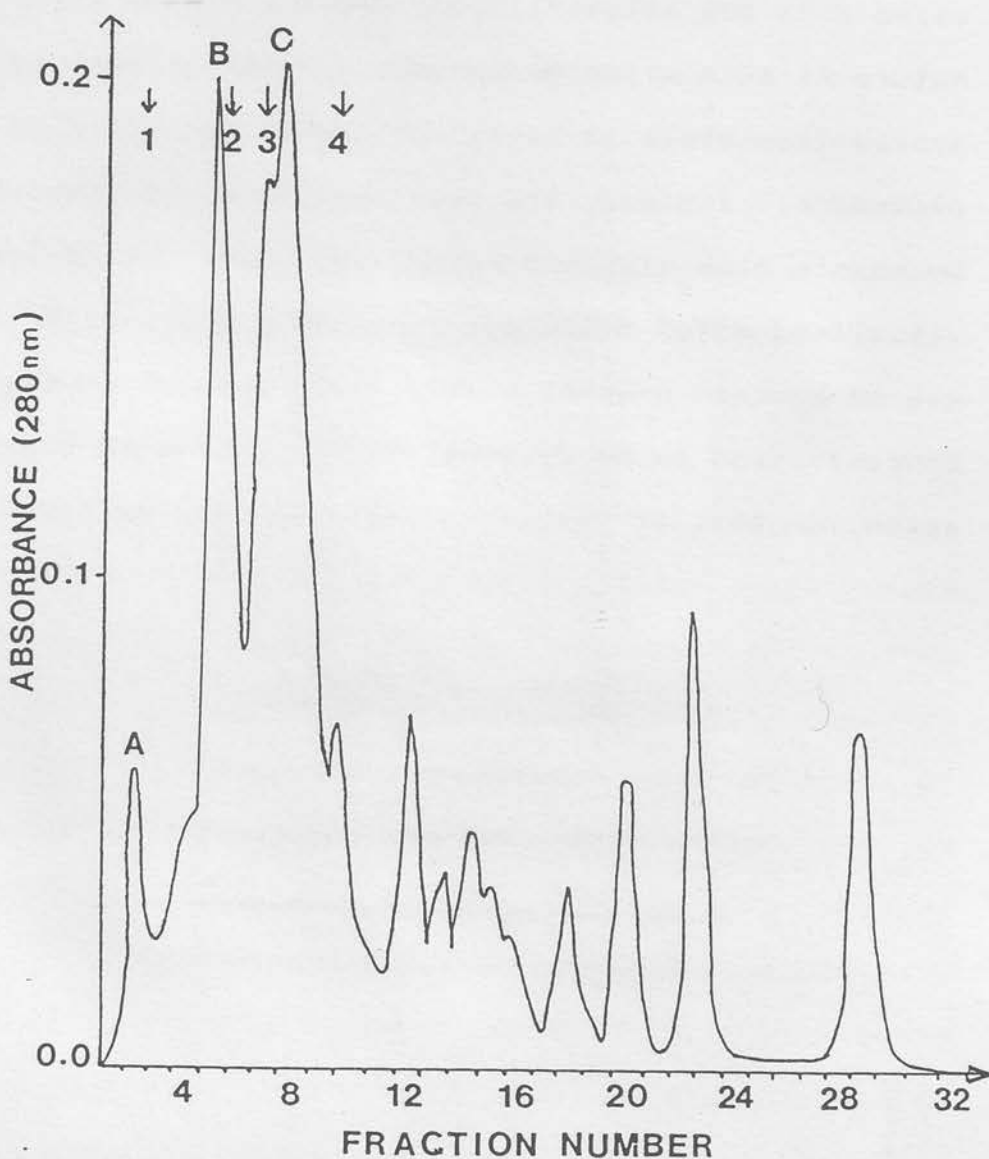


Figure 6.2.12. The 280nm absorbance profile of IPM subjected to a Superose 12 10/30 HR FPLC gel filtration column under native conditions. See text for experimental detail.

- A 1001.A3 antigen
- B IRBP
- C PNA-binding material
- 1 Blue dextran (2000kDalton)
- 2 β -amylase (200kDalton)
- 3 Bovine serum albumin (66kDalton)
- 4 Cytochrome C (12.4kDalton)

Table 6.2.3. The results of dot blotting experiments where the fractions obtained from a Superose 12 HR 10/30 gel filtration chromatography run were dotted onto nitrocellulose and treated with monoclonal antibody or lectin, as described in the text.

Fraction number	Monoclonal antibody (Mab)/lectin				
	no Mab	1001.A1	1001.A3	PNA	ConA
1PM	(-)	(+)	(+)	(+)	(+)
1.	(-)	(-)	(+)	(+-)	(+-)
2.	(-)	(-)	(+)	(+-)	(+-)
3.	(-)	(-)	(-)	(-)	(-)
4.	(-)	(-)	(-)	(-)	(-)
5.	(-)	(+)	(-)	(-)	(+)
6.	(-)	(+)	(-)	(-)	(+)
7.	(-)	(-)	(-)	(+)	(-)
8.	(-)	(-)	(-)	(+)	(-)
9-32.	(-)	(-)	(-)	(-)	(-)

(-) no immunoreactivity/reactivity
 (+-) little immunoreactivity/reactivity
 (+) immunoreactivity/reactivity

6.2.2.6. Gel filtration of IPM in the presence of guanidinium HCl

It was thought possible that the isolated 1001.A3 antigen, shown to have a very high molecular weight by gel filtration under non-denaturing conditions, could be part of a large molecular aggregate of some sort, containing among other things the 1001.A3 antigen, rather than the antigen being a single molecular entity. To test this possibility, IPM was treated with guanidinium HCl and fractionated by gel filtration chromatography in the presence of guanidinium HCl. This is a denaturing agent used to dissociate non-covalently bound material. Molecular aggregates in the presence of guanidinium HCl should dissociate into their primary constituents which would then separate according to their individual sizes by gel filtration chromatography. A 400 ul aliquot of IPM was added to 200 ul of 8M guanidinium HCl. 500 ul of this mixture was subjected to gel filtration chromatography as described in Section 6.2.2.5. except that the system was run in 4M guanidinium HCl instead of TBS (Figure 6.2.13.). Fractions were collected and dot blotted as before to identify which fractions the 1001.A3 monoclonal antibody bound (Table 6.2.4.). The absorbance profiles at 280nm of the gel filtration runs under the denaturing and native conditions were found to be similar (Figures 6.2.12., 6.2.13.). There was no obvious reduction in the amount of the high molecular weight material excluded

from the the gel, in the presence of guanidinium HCl, and there was no obvious increase in material with smaller molecular weights. The results of the dot blotting experiments indicated that the high molecular weight material maintained its ability to bind 1001.A3, although the immunoreactivity was spread over a larger number of fractions. No other fractions corresponding to lower molecular weight material showed an ability to bind 1001.A3 and therefore it was concluded that the high molecular weight material must be a single entity with a molecular weight of at least 2×10^6 Daltons, rather than being a molecular aggregate of smaller components.

Those fractions with the ability to bind 1001.A3 were subjected to SDS polyacrylamide gel electrophoresis. The gel was then silver stained for protein but again no protein was visualised.

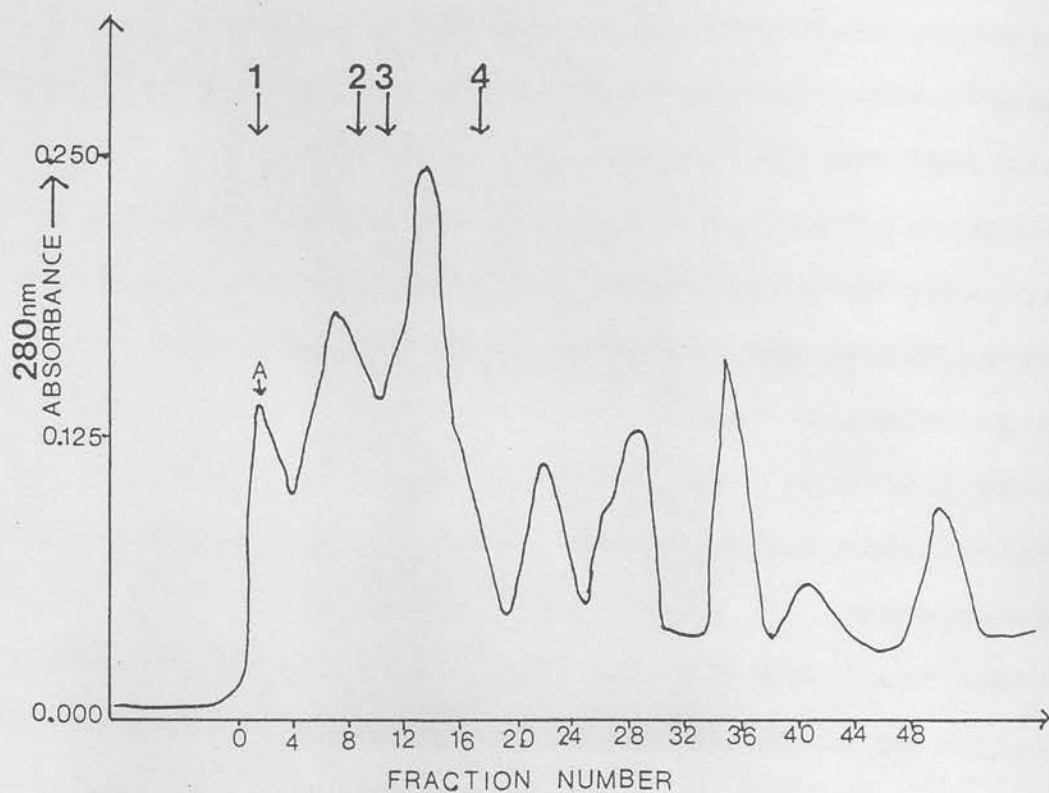


Figure 6.2.13 The 280nm absorbance profile of IPM subjected to a Superose 12 10/30 HR FPLC gel filtration column under denaturing conditions. See text for experimental detail.

- A 1001.A3 antigen
- 1 Blue dextran (2000 KDalton).
- 2 β -amylase (200 KDalton).
- 3 Bovine serum albumin (66 KDalton).
- 4 Cytochrome C (12.4 KDalton).

6.2.3.7. Trypsin treatment of IPM

Trypsin is a serine protease which cleaves peptide bonds on the carboxyl side of arginine and lysine residues (Brown and Kold 1973).

50 µl aliquots of IPM (1 µg/ml or protein) were incubated with trypsin at various concentrations (1 µl/ml-100 µl/ml,

Table 6.2.4 The results of dot blotting experiments where the fractions obtained from a Superose 12 HR 10/30 gel filtration chromatography run under denaturing conditions were dotted onto nitrocellulose and treated with 1001.A3, as described in the text.

Fraction number	1001.A3	no Mab
IPM	(++)	(-)
1.	(+)	(-)
2.	(++)	(-)
3.	(++)	(-)
4.	(++)	(-)
5.	(+)	(-)
6.	(+-)	(-)
7.	(+-)	(-)
8.	(+-)	(-)
9-48.	(-)	(-)

(-) no immunoreactivity

(+-) little immunoreactivity

(+) immunoreactivity

(++) strong immunoreactivity

Mab monoclonal antibody

antibody (Mab)	(100)	(10)	(1)	(0.1)	(0.01)
1001.A1	(-)	(-)	(-)	(+)	(+)
1001.A3	(-)	(-)	(-)	(+)	(+)
no Mab	(-)	(-)	(-)	(-)	(-)

(+) immunoreactivity

(-) no immunoreactivity

(+-) little immunoreactivity

6.2.2.7. Trypsin treatment of IPM

Trypsin is a serine protease which cleaves peptide bonds on the carboxyl side of arginine and lysine residues (Brown and Wold 1973).

50 ul aliquots of IPM (1mg/ml of protein) were incubated with trypsin at various concentrations (1U/ml-100U/ml, final concentration) for 10 minutes at room temperature. PMSF was then added to a final concentration of 0.1mM, to prevent any further protease activity. Immunodotting was carried out using the digested IPM as the antigen solutions. The dots were treated to visualise the binding of either 1001.A1 or 1001.A3 to the digested IPM. Neither monoclonal antibody appeared to bind to the IPM which had been treated with trypsin at a concentration of greater than 10U/ml (Table 6.2.5.).

Table 6.2.5 The effects of trypsin treatment of IPM, with respect to its ability to bind the monoclonal antibodies 1001.A1 and 1001.A3

Monoclonal antibody (Mab)	Concentration of trypsin (U/ml)				
	(100)	(50)	(10)	(5)	(1)
1001.A1	(-)	(-)	(+-)	(+)	(+)
1001.A3	(-)	(-)	(+-)	(+)	(+)
no MAb	(-)	(-)	(-)	(-)	(-)

(+) immunoreactivity

(-) no immunoreactivity

(+-) little immunoreactivity

The IPM treated with the highest concentration of trypsin which had no effect on the immunoreactivity of 1001.A3 antibody (5U/ml), and IPM treated with the lowest concentration of trypsin which abolished the immunoreactivity of the 1001.A3 monoclonal antibody (10U/ml) were subjected to SDS-Polyacrylamide gel electrophoresis (6-15% exponential acrylamide gradient) and Western blotting. The nitrocellulose treated with 1001.A3 showed the high molecular weight material which binds 1001.A3, to have been present in the stacking gel of the gel-track loaded with IPM treated with the low concentration of trypsin whereas it was absent from the gel-track loaded with IPM treated with the high concentration of trypsin. No lower molecular weight degradation products with antibody immunoreactivity were visualised on the nitrocellulose. The procedure was repeated with the high molecular weight material, (isolated by gel filtration) (6.2.2.5.), which binds 1001.A3. The silver stained gel of the trypsin treated fractions failed to visualise the presence of any protein and a similar gel stained by the PAS method failed to detect any carbohydrate present. In conclusion, the IRBP (1001.A1 antigen) and the 1001.A3 antigen present in the IPM both have a protein component which when enzymatically digested causes the structure of the antigenic determinant to change so that it is no longer recognised by, and so no longer binds to the antibody.

It was thought that treatment of the IPM with trypsin would digest the high molecular weight substance which binds 1001.A3, producing degradation products of lower molecular weights with the ability to enter the separating gel in SDS polyacrylamide gel electrophoresis. It was hoped that these could then be detected by staining the gel (for example for protein or carbohydrate). This was not the case and so the substance in the IPM which binds to 1001.A3 remains elusive to detection by any staining procedure other than that based on its antibody binding capacity. It is possible that this substance contains only a very small proportion of protein and that this protein may itself (especially after treatment with trypsin) have a very small molecular weight and therefore may not be detected by this gel electrophoresis system. On the other hand it is possible that the protein may be very large and be effectively unable to enter into the gel systems used.

Further characterisation of this substance would rely on finding its susceptibility to other enzymatic treatments and the use of this information to determine its chemical composition.

The protein content of the 1001.A3 antigen fraction obtained by gel filtration was assayed by the Bradford method and was found to be less than 1 ug/ml. IPM is known to contain a small proportion of glycosaminoglycan and proteoglycan (2% w/w), (Adler and Kluckznif 1982),

some of which are localised to different regions of the IPM (Feeney 1973). For this reason, IPM was treated with enzymes which react with different types of proteoglycan structures to establish whether the substance in the IPM which reacts with 1001.A3 is in fact a proteoglycan. IPM was treated with chondroitinase ABC, hyaluronidase, heparinase or nitrous acid before being tested for immunoreactivity with 1001.A3.

6.2.2.8. Chondroitinase ABC digestion of IPM

Chondroitinase ABC catalyses the release of chondroitin sulphate and dermatan sulphate side chains from proteoglycans giving protein enriched core molecules still containing core side chains (Oike et al 1980, Oike et al 1982). The enzyme has high specificity for galactosamine glycan chains and has no activity on the core proteins, keratan sulphate chains, heparin or heparan sulphate chains. A reduction in antibody immunoreactivity as a result of chondroitinase ABC action on IPM would suggest the presence of chondroitin sulphate or dermatan sulphate groups within the structure of the antigen.

A 50 ul aliquot of IPM was treated with chondroitinase ABC (4.8.4.) and the mixture was tested for its ability to bind the antibodies 1001.A1 and 1001.A3 by the dot incubated under the same conditions without the addition of the chondroitinase ABC maintained its immuno-

blotting method. 1001.A1 showed immunoreactivity with the chondroitinase ABC-treated IPM whereas 1001.A3 did not (Table 6.2.6.).

These results suggest the presence of chondroitin sulphate or derivate sulphate groups within the antigen.

Table 6.2.6 The effects of various enzyme treatments of IPM with respect to its ability to bind the monoclonal antibodies 1001.A1 and 1001.A3.

Monoclonal antibody	Enzymatic treatment of IPM			
	Chon	Hyal	N/A	Hep
(Mab)				
1001.A1	(+)	(+)	(-)	(+)
1001.A3	(-)	(+)	(-)	(+)
no Mab	(-)	(+)	(-)	(-)

(-) no immunoreactivity
 (+-) little immunoreactivity
 (+) immunoreactivity

Chon Chondroitinase ABC
 Hyal Hyaluronidase
 N/A Nitrous acid
 Hep Heparinase

Western blotting of the chondroitinase ABC-treated IPM was carried out and the nitrocellulose was treated to visualise antibody binding. 1001.A3 failed to bind as no staining was seen on the nitrocellulose corresponding to the high molecular weight antigen or to any lower molecular weight degradation products. The IPM incubated under the same conditions without the addition of the chondroitinase ABC maintained its immuno-

reactivity with both antibodies, indicating that this loss of immunoreactivity was due to the enzymatic action of the chondroitinase ABC. These results suggest the presence of chondroitin sulphate or dermatan sulphate groups within the antigen structure. The fact that IRBP was unaffected by the chondroitinase ABC treatment suggests that protease activity in the incubation mixture is not responsible for the loss of antigenicity of the IPM and that this is due to the specific activity of the chondroitinase ABC. The inability of 1001.A3 to bind its antigen after the sulphate groups have been removed from this structure indicates that these sulphate groups may form part of the antigenic determinant.

6.2.2.9. Nitrous acid deamination of IPM

Nitrous acid treatment of the glycosaminoglycan chains of a proteoglycan may result in the cleavage of carbohydrate units at those glycosidic linkages involving hexosamine residues in which the amino groups are either N-sulphated or unsubstituted (Borders and Raftery 1968).

A 50 μ l aliquot of IPM was treated with nitrous acid (4.9.5.) and then processed as for the chondroitinase ABC-treated IPM (6.2.2.8.).

The ability of the IPM to bind 1001.A1 or 1001.A3 was abolished by the nitrous acid treatment (Table 6.2.6.). 1001.A1 would appear to bind the protein structure of

IRBP (since binding is abolished by the action of trypsin) and therefore binding of 1001.A1 to IRBP should not be affected by the nitrous acid treatment, since this should only alter the carbohydrate part of IRBP. It must be assumed therefore that the antigen-treated determinant of IRBP for 1001.A1 was lost due to the harsh acidic conditions imposed on it rather than due to the specific chemical reaction taking place. The same conclusion may be drawn from the loss of 1001.A3 antigenicity of the IPM and therefore no information is gained.

6.2.2.10. Hyaluronidase (testicular) treatment of IPM

Hyaluronidase catalyses the hydrolysis of $\beta(1 \rightarrow 4)$ linkages between N-acetylglucosamine and D-glucuronate in hyaluronic acid (Dorfman 1955). 50 μ l of IPM was treated with hyaluronidase (testicular) (4.9.6.) and then as for the chondroitinase ABC treated IPM (6.2.2.8.). Both antibodies appeared to maintain immunoreactivity with the hyaluronidase treated IPM as seen by the dot blotting method. The control dots which were treated under identical conditions (except that the monoclonal antibody solution was omitted), also stained (Table 6.2.6.). From this it was concluded that the hyaluronidase was binding non-specifically to either the second antibody (anti mouse-biotin conjugate) or to the streptavidin-HRP conjugate during the staining

procedure. This meant that it was impossible to conclude anything with respect to the structure of the 1001.A3 antigen by this method. Western blotting was then carried out and the nitrocellulose blotted with the hyaluronidase-treated IPM failed to stain in the region corresponding to the 1001.A3 antigen in the IPM, or in any other area previously unstained, whereas the control strip of nitrocellulose containing untreated IPM, did stain. From this it was concluded that the 1001.A3 antigen is susceptible to hyaluronidase and that the antigenic determinant may be part of a hyaluronic acid molecule.

6.2.2.11. Heparinase treatment of IPM

Heparinase catalyses the hydrolysis of O-Sulphate and sulphamide groups to produce AB-unsaturated uronides (Linker and Hovingh 1972). A 50 ul aliquot of IPM was treated with heparinase (4.9.3.) and then as for the chondroitinase ABC treated IPM (6.2.2.8.). The heparinase had no effect on the binding of either 1001.A1 or 1001.A3 to IPM (Table 6.2.6.). It was concluded from this that either the 1001.A3 antigen is not a heparan sulphate or heparin molecule, or that the antigenic determinant within such a molecule type is not itself altered by the heparinase treatment.

6.2.2.12. Enzymatic treatment of tissue sections of bovine neural retina

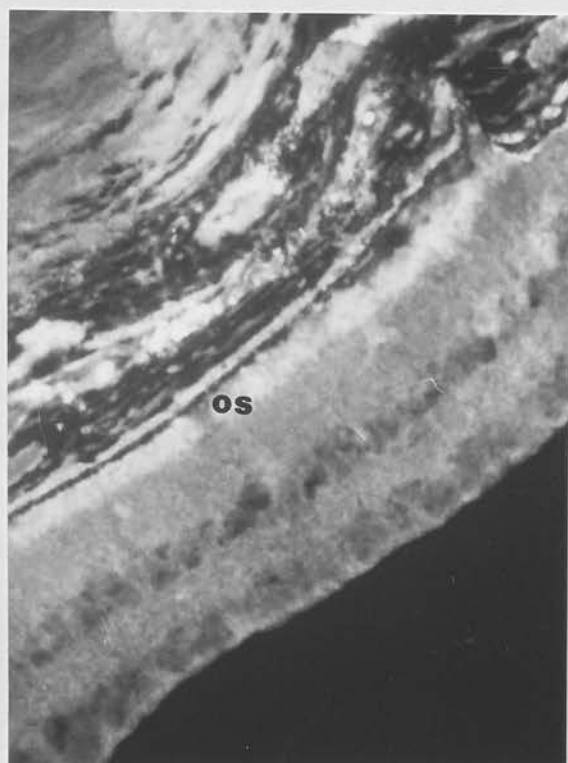
In order to further characterise the nature of the 1001.A3 antigen, the experiments described in Sections 6.2.2.7.-6.2.2.11. were repeated and expanded upon, using fixed tissue sections of the bovine neural retina in place of IPM, and a larger selection of enzymes. Sections were treated with either chondroitinase ABC, chondroitinase AC, hyaluronidase (testicular), hyaluronidase (Streptomyces), heparinase, neuraminidase or trypsin, before being treated to visualise 1001.A3 binding.

Binding of the antibody was unaffected by treating the sections with heparinase or neuraminidase and was completely abolished by treating the sections with either trypsin, chondroitinase ABC, chondroitinase AC or hyaluronidase (testicular). Treatment with hyaluronidase (Streptomyces) had no effect on the ability of the antibody to bind, although the distribution of binding was altered. The sheath structures surrounding the photoreceptors appeared to collapse, giving the immunofluorescence staining a "fuzzy" appearance (Figure 6.2.14.).

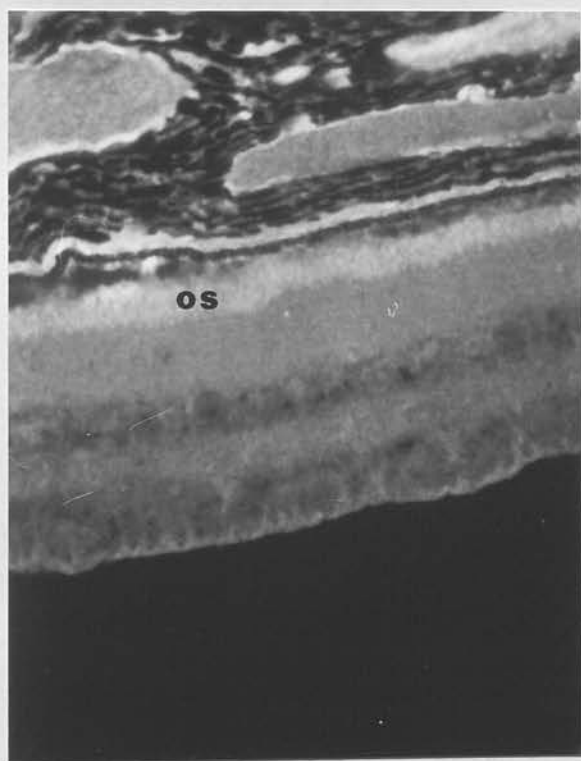
Figure 6.2.14. Photomicrographs of the bovine neural retina treated with enzyme followed by treatment with 1001.A3 antibody, a biotinylated second antibody and Streptavidin-FITC as indicated in the text.

- a. Transverse tissue section of the bovine neural retina treated with chondroitinase AC. Staining of the 1001.A3 antibody is abolished from the photoreceptor cells and the Interphotoreceptor Matrix. (magnification X 150)
- b. Transverse tissue section of the bovine neural retina treated only with biotinylated second antibody and Streptavidin-FITC, indicating background staining in the absence of 1001.A3, similar to that in the chondroitinase AC treated retina. (magnification X 150)
- c. Transverse tissue section of the bovine neural retina treated with Streptomyces hyaluronidase. Staining does not appear to be reduced although the binding pattern is altered. (magnification X 150)
- d. Transverse tissue section of the bovine neural retina treated as in c. The staining pattern of 1001.A3 remains associated with the photoreceptor cells and with the Interphotoreceptor Matrix but is no longer present as an ordered structure and staining adopts a "fuzzy" appearance. (magnification X 900)

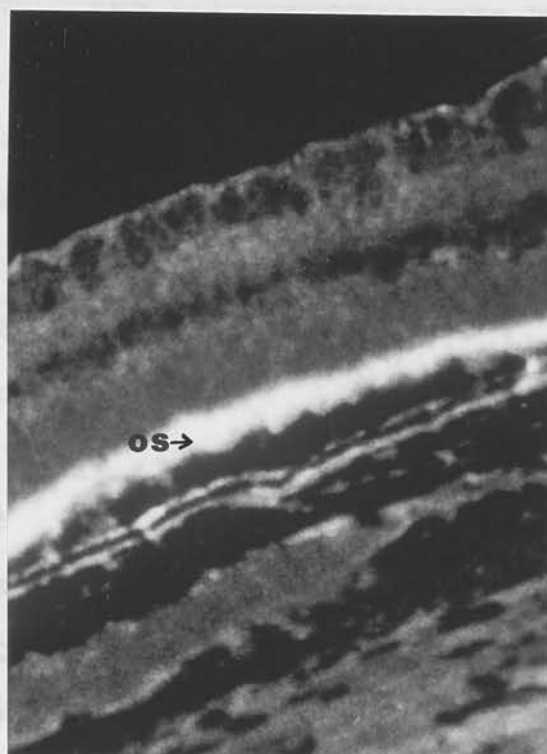
a



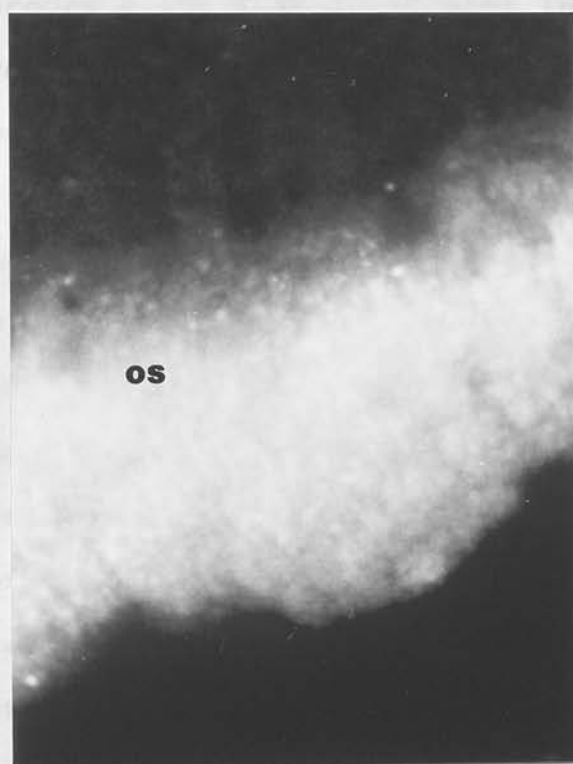
b



c



d



6.2.2.13. Conclusions from the enzymatic treatments of IPM

The fact that the 1001.A3 antigen is sensitive to chondroitinase ABC and chondroitinase AC would suggest it to be a chondroitin A sulphate and/or a chondroitin C sulphate-containing molecule and not a dermatan sulphate molecule, which would not have been affected by the chondroitinase AC enzyme.

The 1001.A3 antigen is sensitive to trypsin, indicating the presence of a core protein and/or link protein, and that it is not a hyaluronic acid molecule (Laurent and Fraser 1986). The sensitivity of the antigen to hyaluronidase (testicular) and not to hyaluronidase (Streptomyces) would appear to verify this.

Hyaluronidase (testicular) as well as using hyaluronic acid as a substrate is capable of using chondroitin sulphate groups A and C as substrates (Borders and Raftery 1968) whereas hyaluronidase (Streptomyces) reacts specifically with hyaluronic acid molecules. The ability of the hyaluronidase (Streptomyces) to alter the binding pattern of 1001.A3 suggests that the antigen is associated with hyaluronic acid molecules and that the chondroitin sulphate-containing molecules, recognised by 1001.A3, are held in a rigid sheath-like structure by the hyaluronic acid molecules.

Evidence exists for the presence of chondroitin sulphate molecules and the absence of dermatan sulphate molecules in the inner segment/outer segment region of the IPM

(Porrello and Lavail 1986). This appears to confirm the conclusions obtained from the analyses of the 1001.A3 antigen structure.

6.2.2.14. 1001.A3 binding to other tissue types

1001.A3 was tested for its ability to bind ovine cartilage and mast cells (which contain and secrete large amounts of proteoglycan) by Dr. H Miller, Moredun Research Unit, Edinburgh. There was no antibody binding, indicating the absence of the 1001.A3 antigen in these cell types.

6.2.2.15. Summary of the 1001.A3 antigen analysis

1001.A3 binds to a component of the IPM which is part of a sheath-like structure, surrounding the inner and outer segments of the photoreceptor cells.

The antigen has an apparent molecular weight of at least 2×10^6 Da and is a chondroitin sulphate-containing molecule, which may be unique to the IPM of the retina. These chondroitin sulphate-containing molecules are associated with hyaluronic acid molecules which are necessary for the maintenance of the sheath structure. The fact that the antibody binding relies on the presence of sulphate groups on the side chains of the presumptive glycosaminoglycans suggests that it is the extent or the positioning of these sulphate groups which makes this molecule unique to the IPM.

6.2.3. Discussion

In retrospect, the methods used to identify the antigens of the 1001.A1 and 1001.A3 monoclonal antibodies were not the most suitable for the task, especially in the case of the 1001.A3 antigen.

Immune precipitations, SDS-polyacrylamide gel electrophoresis and Western blotting techniques are most suitable for the analysis of protein molecules. Such molecules tend to be globular in shape and have a molecular weight of under 200 000 Daltons. These techniques are not as well suited to the analysis of very large molecules or highly glycosylated proteins. The progress of large molecules is hindered through polyacrylamide gels and glycoproteins or proteoglycans are not well detected using conventional protein stains. Such molecules also fail to transfer well onto nitrocellulose from polyacrylamide gels. In order to precipitate the antigen using a monoclonal antibody, there must be high affinity binding between the two molecule types. This is especially true for very large antigen molecules. Higher affinity antibodies may be produced by lengthening the period of time in which mice were immunised, and by subjecting the animals to a greater number of immunisations. Both monoclonal antibodies produced were of the IgM class which tend to have a lower affinity than IgG molecules and do not mature through time (Gearhart et al 1981). The

production of IgG antibodies may be more useful since higher affinity antibodies may be used more successfully in immune precipitations. *be established first.*

Standard biochemical separation techniques such as gel filtration and ion exchange chromatography are also not always suitable, due to the highly charged nature and large size of proteoglycan molecules. Other techniques such as density centrifugation and chemical analyses are often used to characterise such molecules.

The alternative approach to identifying the 1001.A3 antigen and of discovering more about its properties would be to study its biosynthetic pathway. This would be possible using radio-labelled sulphate, amino acid and/or sugar molecule precursors, combined with the use of pathway inhibitors such as β -xylosides (which block the addition of chondroitin sulphate chains to the core glycoprotein by acting as acceptors for the synthesis of sulphated glycosaminoglycan chains) (Schwartz et al 1974a, Schwartz et al 1974b); or tunicamycin (which inhibits the addition of asparagine-linked carbohydrate groups to the protein) (Schwartz et al 1982, Schwartz and Datema 1988). Analysis of the production of the proteoglycan antigen could then be achieved by a combination of immunohistochemistry and sub-cellular fractionation techniques. This it should be stressed is a long-term approach and would not be achieved in a short period of time. An initial problem is that it is not known for sure where the 1001.A3 antigen is

synthesised (the photoreceptor cells, glial cells or pigmented epithelium cells are all possible candidates) and so this would have to be established first.

In conclusion, two monoclonal antibody-secreting cell lines were produced by immunising mice with unpurified mixtures from the retina.

In an attempt to produce more photoreceptor, or IPM-specific monoclonal antibodies, the second strategy (as described in Section 6.1.) was adopted.

RPE

**outer
segments**

**Inner
retina**

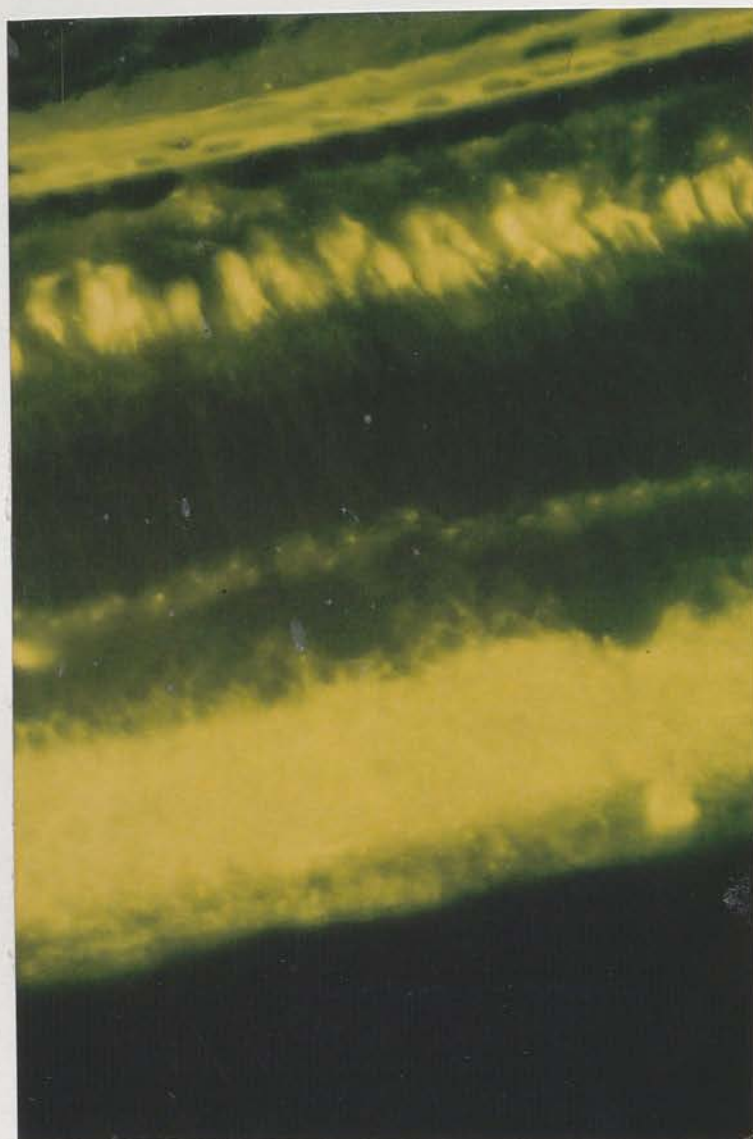


Figure 6.3.1. Fixed tissue section of the bovine neural retina treated with PNA-FITC.

6.3. Immunisation of mice with purified antigen

6.3.1 Introduction

As discussed in Section 6.1., a second approach to the production of cell-specific monoclonal antibodies is to purify, or at least partially purify, a cell-specific antigen with the intention of using this to immunise mice. This would also reduce the probability of producing monoclonal antibodies with similar binding characteristics to those already produced and which are discussed in Section 6.2.

It would be unwise to isolate a substance, for example, a protein, and to assume this to be specific to a particular cell type (in vivo), unless it can be identified both in the in vivo and in vitro situations. The cone photoreceptor cells in the retina are not as well defined biochemically as the rods (discussed in Section 1.6), however the Peanut Agglutinin (PNA)-binding material which is associated with the outer segments of the cones is a candidate suitable for isolation as a cone-specific antigen. This is easily identifiable, since no other substance present in the outer retina binds PNA (as determined by treating tissue sections of neural retina with PNA (2.2.3.)(Figure 6.3.1.)). PNA binds to the inner plexiform layer of the retina as well as to the outer segments of the cone photoreceptors and the cone pedicles. Other lectins such as ConA and WGA also bind the outer segments of the

photoreceptor cells but none specifically to the cones. Treatment of a section of the retina with neuraminidase (which removes terminal sialic acid molecules from the carbohydrate groups of glycoconjugates), followed by treatment of the tissue with PNA, results in the staining of both the rod and the cone photoreceptors. It is not clear if this post-neuraminidase staining is due to the lectin binding to the same molecule, present on both rod and cone cells but normally sialylated when associated with the rods, or whether the PNA is binding to quite different molecules in each case. Substances isolated from the outer retina which bind PNA before neuraminidase treatment must therefore originate from molecules associated with the cone photoreceptors.

6.3.2. Isolating the PNA-binding material

The PNA-binding material is extracellular and forms a domain around the cone photoreceptors, distinct from the remainder of the IPM (Johnson et al 1986). It was necessary to establish whether this material is removed from the retina during the preparation of IPM (5.1.), or whether it remains associated with the retina. To test this, after the removal of IPM, the retina was cut into squares (approximately 25mm² and then embedded, sectioned (2.1.1.) and treated with PNA. The intensity of fluorescence due to the lectin binding appeared the same as in tissue sections of control retina in which IPM had not been removed.

In conclusion therefore, the PNA-binding material of the associated with the cone photoreceptors (or with the inner retina) was not removed from the retina by the normal method of preparing IPM, or at least not to any great extent (it was impossible to tell if a small proportion of the PNA-binding material had been cone removed).

The IPM therefore may contain a small amount of the PNA-binding material originally associated with the cone photoreceptor cells, but the majority of it remains associated with the retina after IPM is removed.

The first step in the purification of this PNA-binding substance would appear to be the removal of IPM from the retina.

6.3.3. Liberating PNA-binding material from the retina using high salt concentrations

The next step in purifying the PNA-binding material associated with the cone photoreceptor cells was to liberate the material from the surface of the retina.

It was important to keep separate any PNA-binding material released from the inner retina from that released from the outer retina. The two are distinct in tissue sections treated to visualise PNA binding (Figure 6.3.1.), but it would be impossible to distinguish them in solution as the characteristics of these molecules

retina did not remove the PNA-binding material associated with the cone photoreceptors. Incubations of

are not known. This meant that the outer surface of the retina had to be treated independently of the inner surface when liberating the PNA-binding material. Attempts were made to isolate the material by subjecting the outer surface of the retina to various high salt concentrations, in order to release it from the cone plasma membrane with which it is associated. IPM was removed and the retina was placed face down in a Petri dish (with the photoreceptors facing downwards), so that it was floating on the incubation solution. Incubation solutions containing high salt concentrations were used in an attempt to remove the PNA-binding material associated with the cone photoreceptors, as such conditions have been used to liberate other membrane-associated, or extrinsic membrane proteins from membranes (Howell and Palade 1982, Higgins 1984). The retina was floated in 1-2ml of either 50mM Tris HCl pH 7.4 containing 500mM sodium chloride, or 100mM sodium carbonate/sodium hydroxide pH 11, on ice for periods of up to 30 minutes. The retina was then rinsed in a large volume of TBS before being embedded and sectioned (2.1.2.). The sections were treated with PNA. Fluorescence was as intense as for the control retina sections (obtained from retinae treated identically, except that the incubation solution was TBS). In conclusion, the high salt concentration treatment of the retina did not remove the PNA-binding material associated with the cone photoreceptors. Incubations of

the retinae for longer periods of time resulted in the tissue becoming very gel-like in appearance and difficult to handle, so an alternative treatment was required.

6.3.4. Treatment of the surface of the retina with trypsin

The outer surface of the retina was treated enzymatically with trypsin, by the method described above for high salt concentration treatment, except that the retina was incubated in 1ml of TBS, containing 0.001% (w/v) trypsin (10U/ml) for 10 minutes at room temperature. Care was taken not to expose the inner surface of the retina to this solution. The retina was then drained of excess liquid and rinsed in a large volume of TBS containing 0.1mM PMSF to prevent further protease activity (Provyty and Goldberg 1972). PMSF was also added for the same reason to a final concentration of 0.1mM to the remaining incubation solution. The retina was then embedded and sectioned and the sections treated with PNA. No fluorescence was visible in the outer retina. The PNA-binding material associated with the cone photoreceptors had most probably been liberated from the retina into the remaining incubation solution. This solution was retained for analysis to determine whether PNA-binding material was indeed present.

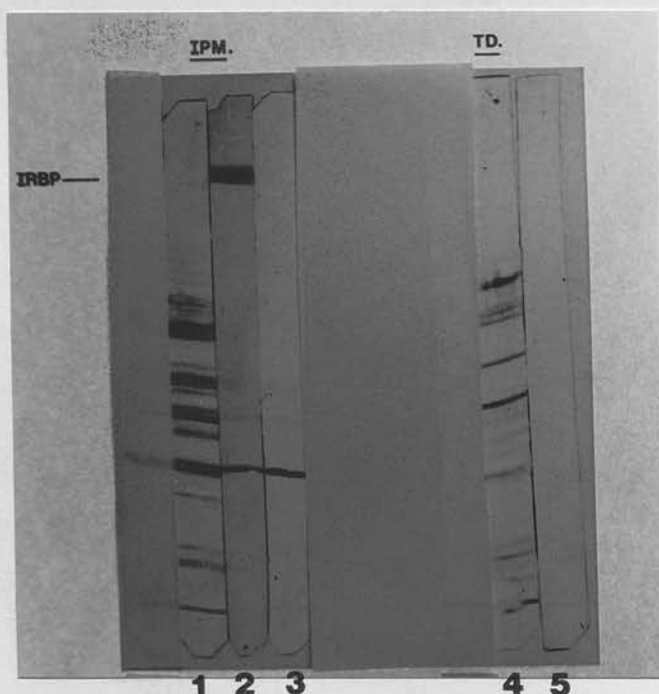


Figure 6.3.2. Strips from a Western blot of a 10% polyacrylamide gel loaded on one half with 300ug IPM and on the second half with 300ug trypsin digest. The strips were treated with biotinylated lectins followed by Streptavidin-HRP. See text for experimental detail.

1. IPM treated with PNA.
2. IPM treated with ConA.
3. IPM treated with Streptavidin only.
4. Trypsin-digest treated with PNA.
5. Trypsin-digest treated with ConA.

It was assumed that the incubation solution would contain some membrane-associated material originating from damaged outer segments and so detergent was used to solubilise protein from the membrane fragments. Triton-X100 detergent was added to the incubation solution (1% (v/v), final concentration), and the mixture allowed to stand on ice for 30 minutes before being centrifuged at 100 000g for 30 minutes. The supernatant was removed, aliquoted into smaller volumes and stored at -20°C.

trypsin-digest which were treated with PNA had similar

6.3.5. Analysis of the trypsin-digest solution containing PNA-binding material

The solubilised fraction, named "trypsin-digest", was assayed for protein by the Bradford assay (4.4.) and was found to contain approximately 1mg/ml of protein. This was dotted onto nitrocellulose and treated with PNA (4.3.4.). The chloronaphthol-stained nitrocellulose was compared to similar dot blots of IPM. The nitrocellulose dotted with trypsin-digest stained the more intensely of the two. This indicated that both solutions contained PNA-binding material and that this was at a higher concentration in the trypsin-digest solution than in the IPM. Western blotting was then carried out using trypsin-digest (4.3.1.) and strips of the nitrocellulose were treated with PNA or ConA (4.3.3.). The chloronaphthol staining pattern on the nitrocellulose indicated the presence of several PNA-binding glycoproteins (Figure 6.3.2.). The

nitrocellulose treated with ConA was devoid of staining, indicating there was no IRBP or digested fragments of IRBP (containing mannose residues) present in the trypsin-digest and this was taken to indicate the absence of other proteins from the IPM or of rod outer segment material which may bind ConA. Western blots of IPM were treated with PNA or ConA (Figure 6.3.2.) and these were compared to those of the trypsin-digest. Those strips of nitrocellulose containing IPM or trypsin-digest which were treated with PNA had similar staining patterns whereas those strips treated with ConA were different.

This suggests that the two solutions have common components, some of which bind PNA, but that they are not identical in composition. It seems that the IPM prepared as described in Section 5.1., contains contaminating proteins from the surface of the retina, which do not constitute part of the interphotoreceptor matrix proper, or which are normally shed into the IPM by the photoreceptor cells. The alternative possibility, that the trypsin-digest contains contaminants from the IPM was ruled out by the lack of IRBP, the major glycoprotein of the IPM, in the trypsin-digest.

This finding explains the source of PNA-binding glycoproteins in IPM and indicates that these are probably the same as the PNA-binding glycoproteins present in the trypsin-digest. Since these PNA-binding

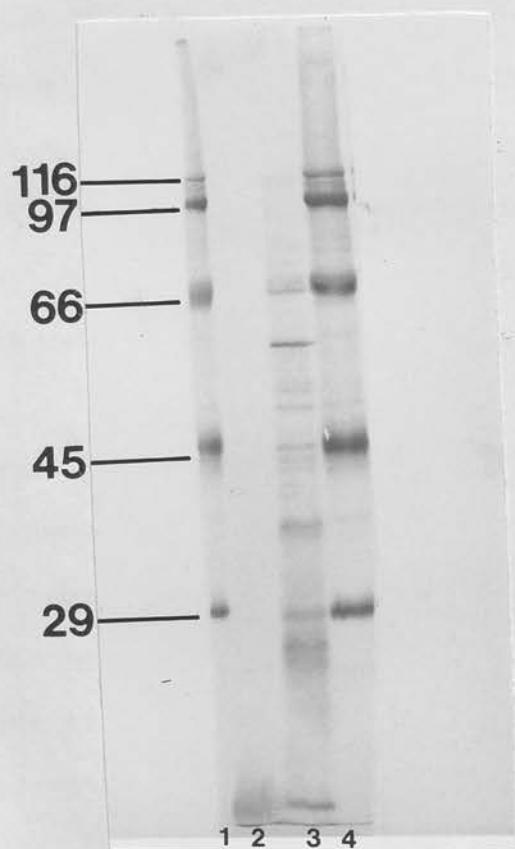


Figure 6.3.3. A silver stained 10% polyacrylamide gel showing the results of PNA-affinity chromatography of Trypsin-digest. See text for experimental detail.

1. Molecular weight markers (15ug).
2. PNA-binding fraction (40ul).
3. Trypsin-digest (starting material) (40ul).
4. as 1.

glycoproteins which are present in the trypsin-digest have been shown to originate from the cone photo-receptors, those PNA-binding proteins present in IPM must also originate from the cone photoreceptors. A polyacrylamide gel (4.1.1.), loaded with aliquots of the trypsin-digest was stained for protein by the silver stain method (4.2.2.)(Figure 6.3.3.). Many proteins with a wide range of molecular weights were visualised. No IRBP was present, confirming the absence of ConA binding in the Western blot. Attempts were therefore made to isolate the PNA-binding glycoproteins from the trypsin-digest. In order to reduce the steric hindrance on the PNA molecules caused by the conjugation process and also to increase the concentration of PNA molecules present on the gel, the affinity chromatography was repeated using a commercial agarose gel conjugated to PNA (Pharmacia). A 100ul aliquot of the agarose-bio gel (capable of binding 1.ug of protein per ul of gel, according to the supplier) was added to approximately 1ml of 50mM Tris and 5mM NaCl. To the solution was added 500ul of 10% (w/v) PNA in 100mM Tris and 100mM NaCl and this was mixed end-over-end, overnight at 4°C. After spin washing, 200ul of PNA conjugated to agarose (4.4.3.2.) was added to the pelleted gel and the mixture incubated end-over-end for 8 hours at 4°C. The gel was spin

6.3.6. Affinity chromatography of trypsin-digest

Affinity chromatography of trypsin-digest was carried out using the batch method, essentially as described in Section 4.8.1. and the mixture incubated for 8 hours end-over-end. A 1ml aliquot of Sepharose-4B conjugate, containing approximately 1mg of lectin, was incubated with 1ml of trypsin-digest (previously dialysed against column buffer). Material which had bound to the gel was eluted in 1ml of column buffer containing 200mM galactose. This fraction was subjected to SDS-polyacrylamide gel electrophoresis which was subsequently silver stained. No protein was apparent in the eluate indicating that none of the PNA-binding material had been affinity purified from the trypsin digest. In order to reduce the steric hindrance on the PNA molecules caused by the conjugation process and also to increase the concentration of PNA molecules present on the gel, the affinity chromatography was repeated using a commercial agarose gel conjugated to biotin (agarose-bio). A 100ul aliquot of the agarose-bio gel (capable of binding 1.1mg of avidin per ml of gel, according to the suppliers) was added to approximately 1ml of Sepharose 4B and spin washed. To the pelleted gel was added 500ul of avidin (1mg/ml in TBS) and this was mixed end-over-end, overnight at 4°C. After spin washing, 200ul of PNA conjugated to biotin (PNA-bio)(4.6.3.2.) was added to the pelleted gel and the mixture incubated end-over-end for 8 hours at 4°C. The gel was spin

washed and 3ml of dialysed trypsin-digest was added to the pelleted gel. After spin washing, 500ul of column buffer containing 200mM galactose was added to the pelleted gel and the mixture incubated for 8 hours end-over-end at 4°C. The gel was spin washed for a final time and stored in excess column buffer containing 200mM galactose and 0.05% (w/v) sodium azide. Aliquots of the original trypsin-digest and of the sugar eluted fraction were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. These were silver stained for protein and treated with PNA respectively. In the lane of the gel which contained the sugar-eluted fraction, a small amount of very low molecular weight material was apparent, very close to the dye front. The nitrocellulose treated with PNA failed to stain. The PNA affinity chromatography procedure was repeated and the PNA-bio solution tested for its lectin content after it was incubated with the gel mixture. Sections of neural retina were treated with PNA using the PNA-bio solution and the fluorescence method. No fluorescence was seen indicating the the PNA-bio had been removed from the solution and was present in the gel mixture. The same results were obtained for the affinity chromatography, none of the PNA-binding glycoproteins being affinity purified from the trypsin-digest. Final attempts to do this were carried out using a commercial PNA-agarose gel (containing 2-4mg of lectin/ml of gel, according to the suppliers). This was

a much higher concentration of PNA than was present in the Sepharose 4B-PNA conjugate. 2ml of trypsin-digest was added to 100ul of the PNA-agarose conjugate and the chromatography was carried out by the batch method. The material which passed through the gel without binding and the material eluted from the gel by the addition of 200mM galactose were each collected in 100ul batches and 50ul aliquots of each were subjected to SDS-polyacrylamide gel electrophoresis and the gel stained for protein by the silver stain method. Those lanes of the polyacrylamide gel containing either the pass-through material which failed bind to the column, or the original trypsin-digest, had similar polypeptide staining patterns. This indicated that both fractions had the same constituents and that nothing in the trypsin-digest appeared to have been removed completely by the affinity chromatography procedure. The lane containing the material eluted with the sugar solution again showed very little staining. The staining corresponded to the same very low molecular weight material as described above which was not obviously apparent in the original trypsin-digest (Figure 6.3.3.). The procedure was repeated and the same result was obtained. It is possible that this very low molecular weight material does have a carbohydrate group containing terminal galactose residues as part of its structure, and to which PNA binds. The affinity chromatography

procedure may have acted to concentrate it more than it is in the original trypsin-digest. The material was apparent in an area of the gel very close to the dye front indicating that it may be a glycolipid rather than a glycoprotein. The fact that this material when visualised by the silver stain method, appeared very yellow in colour (rather than dark brown or grey as most proteins and glycoproteins appear) would support this idea. This would explain why this material was not detected in Western blots, since it would tend to dissolve in the transfer process due to the high methanol content of the blotting buffer. None of the PNA-binding glycoproteins visualised by staining Western blots of the trypsin-digest (as described in Section 6.3.5.) were apparent in this eluted fraction from the trypsin-digest. A higher concentration of other PNA-molecules which bind more strongly to PNA would effectively compete out the binding of the PNA-binding glycoproteins to the immobilised PNA. In conclusion the affinity chromatography procedure was not successful in isolating the PNA-binding glycoproteins from the trypsin-digest. One explanation for the failure is the possibility of steric hindrance on the PNA molecules after conjugation to the agarose and that the galactose residues of the glycoproteins may be unable to bind to the lectin. This would be detected in the IPM (4.3.2.5.) and so those detected in trypsin-digest by the PNA-affinity chromatography may be

especially true for carbohydrate groups which are part of a large molecule, or those which are hindered by other bulky groups.

It is possible that the PNA-binding glycoproteins found in trypsin-digest by SDS-PAGE, are present in vitro in the form of a large, high molecular weight complex of macromolecules which may not dissociate in the conditions used in the PNA-affinity chromatography. Such a complex would tend not to bind to the lectin under these conditions whereas the individual glycoproteins may bind readily, when dissociated. Denaturing conditions may have been required for the dissociation of the complex to allow binding of the glycoproteins to the lectin. In contrast the results from the gel filtration chromatography of IPM in non-denaturing conditions (6.2.2.5.) in which all the PNA-binding material was found to have an apparent molecular weight of 30-70KDaltons, would appear to contradict the idea of a large aggregate of PNA-binding material. One could however argue that the PNA-binding glycoproteins are at some point in time, shed individually into the IPM from a large complex of PNA-binding molecules found only associated with the cone photoreceptor cells. These would therefore be detected in the IPM only in the form of the lower molecular weight glycoproteins. No low molecular weight PNA-binding glycolipids are detected in the IPM (6.2.2.5.) and so those detected in trypsin-digest by the PNA-affinity chromatography may be

tightly associated within the complex in vivo, dissociation only occurring in the presence of trypsin in vitro. From trypsin-digest, mice were immunized. To verify the presence of such a high molecular weight complex of molecules trypsin-digest was treated by Superose 12 HR 10/30 gel filtration chromatography under native conditions in the same manner as was IPM fusions (6.2.2.5.). The high molecular weight material excluded from the gel was tested by dot blotting for its ability to bind PNA. Chloronaphthol staining was found to be very high indicating a large amount of PNA-binding material present. The argument for the presence of a large complex of PNA-binding material would therefore appear feasible. bind PNA in unfixed tissue sections and so these were used to prevent losses in antigenicity within the tissue due to fixation, but no immunoreactivity was detected in the retina and so this fusion was also abandoned.

6.3.7. Immunisation of mice with trypsin-digest

Since the PNA-binding glycoproteins could not be easily isolated from trypsin-digest, mice were immunised (3.1.1.) with trypsin-digest and cell fusions carried out as described in Section 3.1.4. in an attempt to produce monoclonal antibodies against the PNA-binding glycoproteins present in the solution. Two such fusions were carried out. The first was abandoned after 6 days when it became obvious that no colonies of hybridoma cells were present. The second fusion produced colonies of cells in 261 of the 480 wells observed. On Day 10 all the wells were screened using unfixed sections of the neural retina (the cone photoreceptor cells maintain their ability to bind PNA in unfixed tissue sections and so these were used to prevent losses in antigenicity within the tissue due to fixation), but no immunoreactivity was detected in the retina and so this fusion was also abandoned.



Figure 6.3.4. Western blot of a 10% polyacrylamide gel loaded with fractions obtained from a Superose 12 gel filtration column loaded with 500ug IPM. The blot was treated with biotinylated PNA followed by Streptavidin-HRP and stained with chloronaphthol. See text for experimental detail.

1. IPM. (70ug).
2. 60ul fraction 7 (Figure 6.2.12.).
3. 60ul fraction 8 (Figure 6.2.12.).

6.3.8. Isolating PNA-binding glycoproteins from IPM

From dot blotting experiments, IPM appeared to have a lower concentration of PNA-binding constituents than the trypsin-digest, however IPM is more readily available, being much easier to prepare in large volumes than is trypsin-digest. For this reason, attempts were made to isolate the PNA-binding glycoproteins from IPM.

6.3.9. Gel filtration chromatography of IPM

An FPLC Superose 12 10/30 HR gel filtration column was used to fractionate the IPM as described in Section 4.8.4. 1ml fractions were collected, dotted onto nitrocellulose and treated with PNA (4.3.4.). Two fractions were obtained which contained material to which PNA binds with an apparent molecular weight of 30-70Kdaltons (Figure 6.2.12 and Table 6.2.3.). This was distinct from the fractions containing IRBP (identified by the ability to bind ConA and 1001.A1). The PNA-binding material in this fraction was subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting, or protein staining. The nitrocellulose was treated with PNA and the gel stained for protein by the Coomassie blue method. The stained nitrocellulose indicated the presence of several glycoproteins which are capable of binding PNA (Figure 6.3.4.) When compared to a Western blot of IPM and of trypsin-digest which had been treated with PNA (Figure 6.3.2.), the staining patterns of the PNA-binding were similar,

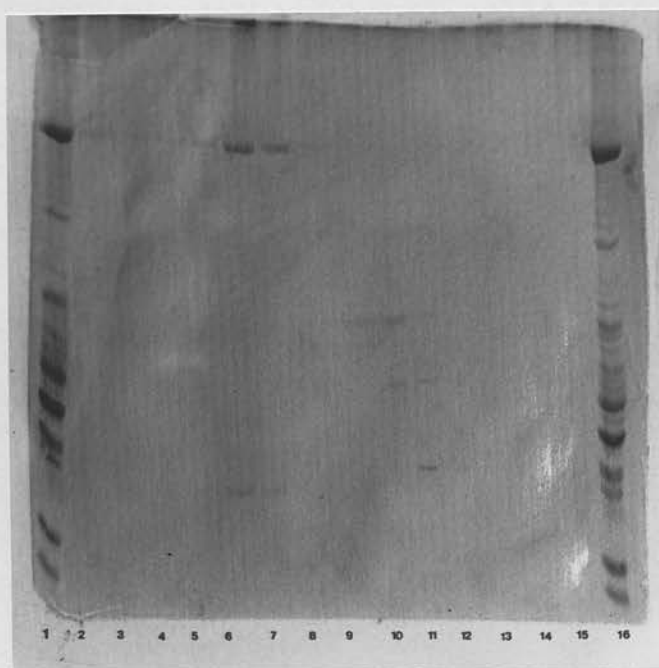


Figure 6.3.5. Coomassie stained 10% polyacrylamide gel visualising fractions obtained from a Superose 12 10/30 HR FPLC column, loaded with 500ug IPM. 50ul fractions were loaded onto the polyacrylamide gel, see text for experimental details.

1. IPM (60ug).
2. fractions 1-14 (Figure 6.2.12.).
3. as 1.

indicating that PNA-binding glycoproteins present in IPM and in the trypsin-digest solution were contained in this gel filtration fraction. On the other hand, the staining patterns were very different when compared for polypeptide bands (Figures 6.2.8., 6.3.3. and 6.3.5.). According to the Coomassie stained gel, the two fractions (obtained by the gel filtration of IPM) containing the PNA-binding material, contained few of the proteins found in IPM and did not appear to contain unique polypeptides (with respect to the other fractions collected from IPM) as may have been expected from the dot blotting experiment. It is possible that the PNA-binding glycoproteins identified by Western blotting, are not visualised by the protein staining method used. Glycoproteins often do not stain well for protein. In conclusion, the PNA-binding glycoproteins present in the IPM were isolated into two gel filtration fractions which were free of the majority of the other proteins present in IPM.

6.3.10. Immunisation of mice with partially purified glycoproteins from IPM

The gel filtration fraction containing PNA-binding constituents (6.3.9.) was used to immunise mice and cell fusions were carried out in an attempt to produce monoclonal antibodies against the PNA-binding glycoproteins found in IPM. Four such fusions were carried out. In the first fusion, of the 576 wells

observed less than 80 wells contained colonies of cells. Those wells which contained cell colonies were screened individually on Day 12 for the presence of anti-retinal antibodies. No fluorescence was apparent on the tissue sections treated with the culture media, indicating the absence of any anti-retina antibodies and so the fusion was abandoned on Day 15. The second fusion was abandoned on Day 5 due to the absence of colonies in the wells and the third fusion was abandoned on Day 6 due to the appearance of fungal contamination in the wells. In the fourth fusion, colonies were observed in most of the wells of the six 96-well plates used and all were screened on Day 10. The majority of the sections treated with culture media had fluorescence across the entire retina at a higher level than present in the control sections. It was impossible to distinguish whether the antibodies detected were all capable of binding to cells across the entire retina or whether antibodies were present which were specific for cell layers within the retina. Such antibodies may have been present and their specificity obscured by the more general binding of any one of the other antibodies in the group of six taken together for the initial screening. It was not practicable to screen each of the 576 wells individually, nor was it practicable to expand each well which may have contained antibody secreting cells. For this reason, 96 of those wells containing the largest colonies were expanded into four 24-well

plates. When screened individually, 7 of these produced a high level of fluorescence throughout the retina and these were expanded into flasks. Further screening revealed no antibody secretion in 2 of the 7, the remaining 5 containing antibody which showed immunoreactivity with all the layers of the retina and so these were finally abandoned on Day 23. From this it would appear that the PNA-binding glycoproteins associated with the cone photoreceptors are not very immunogenic or that the PNA-reactive material contains antigenic epitopes common to most cells of the retina. The isolation of these glycoproteins from those other molecules in IPM which are present in abundance e.g. IRBP, did not appear to improve the antigenic response to this material in the mice immunised with it.

In order to produce a strong immune response, the spleen cell/antigen mixtures were incubated in an atmosphere containing 5% CO₂ at 37°C for 4 days before the fusion was carried out. The spleen cells were pooled, pelleted by centrifugation at 1000g for 10 minutes, and resuspended in 10ml of serum-free medium. The cells were then counted before fusion with the eyecup cells (3,1,4.). Two such

6.3.11. in vitro immunisations

One method of producing an immune response against non-immunogenic material is to allow in vitro priming of the spleen cells before carrying out the cell fusion (Reading 1982).

This was done using the gel filtration fraction (6.3.9.) containing the PNA-binding material isolated from IPM as the in vitro immunogen.

The spleen from a ten week old female Balb/c mouse was aseptically removed. The cells were teased apart and suspended in 15ml of serum-containing medium (3.1.2.). 5ml aliquots of the cell suspension were added to three 25cm² flasks. The antigen mixture was sterilised by filtration through a 0.22um filter before being added to the spleen cells. Different quantities of antigen were added to the three flasks (10, 50 or 100ug), in the hope that the optimum ratio of spleen cells to antigen molecules would be present in at least one of the flasks in order to produce a strong immune response. The spleen cell/antigen mixtures were incubated in an atmosphere containing 5% CO₂, at 37°C for 4 days before the fusion was carried out. The spleen cells were pooled, pelleted by centrifugation at 1000g for 10 minutes, and resuspended in 10ml of serum-free medium. The cells were then counted before fusion with the myeloma cells (3.1.4.).

Two such trypsin-digest treated with PNA (Figure 6.3.2), stain less intensely than might be expected. Although direct

fusions were carried out, the first by Dr J Haywood. In both cases, few colonies were produced in the wells and so both fusions were abandoned.

6.3.12. Discussion

The use of partially-purified cone-specific antigen failed to result in the production of any cone-specific monoclonal antibodies.

The failure to produce antibodies may be explained if the immunisation mixtures were not particularly antigenic. It is often the case that molecules which are highly conserved between species do not elicit an immune response in the injected animal (Goding 1983) and so no antibodies of any use to the research worker are produced.

Another possibility is that the mice did respond to the immunisations and became hyperimmune to the antigens, but that the hybridoma cells produced in the fusion failed to secrete antibody. Cell fusions which produce colonies of hybridoma cells do not necessarily produce colonies of antibody-secreting cells. There is no safeguard against this possibility and a certain amount of luck is involved.

The outer segment of the retina is highly fluorescent after tissue sections are treated with PNA (Figure 6.3.1.). In comparison, the Western blots of the trypsin-digest treated with PNA (Figure 6.3.2), stain less intensely than might be expected. Although direct

comparisons cannot be made between the intensity of staining in the two different systems, one would expect the PNA-binding glycoproteins to be present in abundance in the trypsin-digest (since the PNA-binding material which is present on the retina is removed completely in the procedure used to prepare the trypsin-digest), and the Western blots should therefore stain strongly. It is possible that the glycoproteins which have been isolated form only a minor part of the PNA-binding material on the outer retina. The use of trypsin to liberate this material may cause the glycoproteins to be liberated from a larger complex, itself released from its attachment to the membrane by trypsin. This complex may be comprised of high molecular weight material, or of aggregates of lower molecular weight molecules such as glycolipids. The results of the PNA-affinity chromatography procedure indicated the possibility of the presence of galactose-containing glycolipid in the trypsin-digest. The fact that a material binds PNA indicates that it contains carbohydrate groups with terminal galactose sugar groups. These are usually associated with O-linked carbohydrate groups (rather than N-linked carbohydrate groups). In general, O-linked are less common than N-linked carbohydrates in glycoproteins, but are often found in proteoglycans and glycolipids (Wagh and Bahl 1981).

Be sure of the degree of purity of isolated substances. The immune response to contaminants present in an immunisation mixture even

The trypsin-digest was subjected to gel filtration in native conditions in a similar way to IPM (4.8.4.). This produced a fraction containing very high molecular weight material which was excluded from the gel beads and which stained intensely when dot blotted and treated with PNA. This indicated the presence of high molecular weight, PNA-binding material in the trypsin-digest. Should the trypsin-digest contain a high concentration of PNA-binding material other than the glycoproteins identified in the trypsin-digest and IPM, then these PNA-binding glycoproteins may only constitute a minor proportion of the PNA-binding material associated with the cone photoreceptors. If this is true, then these glycoproteins may be present in the retina in very small quantities, or in very few cells, which would make the detection of antibodies raised against this material very difficult.

In conclusion, the second method discussed in Section 6.1., of immunising mice with purified antigen, for the production of cone-specific monoclonal antibodies was not successful.

PNA-binding material was only partially purified. If antigen free from contaminants is used to immunise mice, in theory all the monoclonal antibodies produced from the subsequent cell fusions will bind the antigen. In practice it is very difficult to be sure of the degree of purity of isolated substances. The immune response to contaminants present in an immunisation mixture even

in very small amounts, is often much higher than the immune response to the substance against which antibodies are desired (Goding 1983), especially if the contaminant is immunogenic. A method of avoiding this is to isolate the immunogen to the extent that it is possible to determine or partially determine its amino acid sequence (this information may be obtained more easily by obtaining the gene sequence). Using this information it would then be possible to construct a synthetic antigen, free from the contaminants which tend to co-purify with the natural antigen.

This use of a synthetic antigen (as discussed in Section 6.1.) was the final approach taken to raise cone-specific monoclonal antibodies.

Figure 6.4. Localization of conserved (black) amino acids in the amino acid sequences of human, bovine and avian rhodopsin. If 4 of the 5 sequences have conserved substitutions and the fifth is nonconserved, the position is considered nonconserved.

Amino acids are considered conserved if they belong to the same class (1-5).

1. Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Cys.

2. Ser, Thr, Cys, Tyr, Asn, Gln.

3. Asp, Glu.

4. Cys, Arg, His.

5. Phe, Tyr, Trp.

6. His, Trp.

(Adapted from Nathans *et al.* 1986)

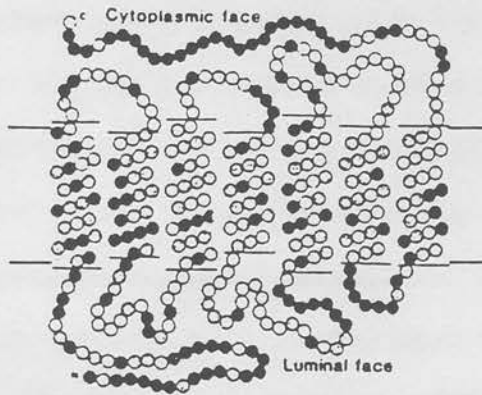


Figure 6.4.1 Localisation of nonconserved (black) amino acids in all four human visual pigments and bovine rhodopsin. If 4 of the 5 sequences have conserved substitutions and the fifth is nonconserved, the position is considered nonconserved. Amino acids are considered conserved if they belong to the same class (1-5).

1. Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Cys.
2. Ser, Thr, Cys, Tyr, Asn, Gln.
3. Asp, Glu.
4. Cys, Arg, His.
5. Phe, Tyr, Trp.
6. His, Trp.

(Adapted from Nathans et al 1986)

6.4. Immunising mice with pure antigen (an approximate ratio of one to thirty) (Marc and Sperling 1977, McDermott)

6.4.1. Introduction

The production of cone-specific monoclonal antibodies should be possible by immunising mice with a pure protein, or a peptide fragment of a pure protein, unique to these cells and by taking the spleen of the immunised mouse to carry out a cell fusion to produce the cell-specific monoclonal antibodies.

The sequence of the bovine rhodopsin gene has been determined (Nathans and Hogness 1984) and this has been used to construct oligonucleotide probes in order to find homologous sequences in the human genome (Nathans et al 1986). The human rhodopsin gene was found to have very high homology with its bovine counterpart, (96% amino acid identity) and three other genes were found which have lower homology (43% amino acid identity). These were identified as the genes encoding the three cone pigment proteins present in the red, blue or green cones. Hydropathy plots suggest the four gene products have similar structures in the membrane and vary most at their amino terminus, present in the lumen of the disc. (Nathans et al 1986)(Figure 6.4.1).

Those cDNA clones constructed for the blue pigment protein had a frequency one hundred and fifty times lower than that of the rhodopsin cDNA clones (Nathans et al 1986), which corresponds approximately to the ratio of blue cones to rods in the human retina (compared to

the red/green cones and rods which have an approximate ratio of one to thirty) (Marc and Sperling 1977, McCrane et al 1983).

The eleven N-terminal amino acids of the red and green pigment proteins are identical, but differ from the corresponding amino acid sequence of the blue pigment protein and of rhodopsin (Nathans et al 1986). The higher proportions of red/green cones to blue cones means that there would be a greater probability of detecting red/green cone-specific antibodies than blue cone-specific antibodies. It was hoped that monoclonal antibodies raised against a synthetic peptide, corresponding to the N-terminus of the red/green pigment proteins would also show immunoreactivity against the native protein in vivo, associated with cone photoreceptor cells. The N-terminus of the rhodopsin molecule is different to that of the red/green pigment proteins and therefore monoclonal antibodies with immunoreactivity against the former should distinguish between these and the rod-specific protein. This was the approach taken attempt to raise cone-specific monoclonal antibodies.

6.4.2. Synthesis of the antigen

An undecapeptide corresponding to the N-terminus of the red and green pigment proteins (N-ALA-GLN-GLN-TRP-SER-LEU-GLN-ARG-LEU-ALA-GLY), was synthesised using the t-BOC method on an LKB automatic peptide synthesiser, and was deprotected and removed from the resin using trifluoroacetic acid. The resulting peptide mixture was subjected to HPLC using a C14 reverse phase column and the major peak was collected. This was carried out by Dr R. Kelly of the MRC Reproductive Biology Unit, Edinburgh.

The purified peptide was subjected to amino acid analysis and was sequenced, to check that the correct structure had been synthesised and purified.

Table 6.4.1 Amino acid analysis of the synthetic peptide corresponding to the N-termini of the red and green cone pigment proteins.

The retention times of the peptide amino acids are compared with those of the standard amino acids and the relative amounts of each amino acid used to calculate the empirical ratio of each within the peptide.

See text for experimental detail.

<u>Standard amino acids</u>		<u>Peptide amino acids</u>		
retention time (minutes)	amino acid	retention time (minutes)	% area under peak	empirical ratio
12.33	ASP	(-)	(-)	(-)
14.34	THR	(-)	(-)	(-)
15.16	SER	15.19	2.045	1
17.39	GLN	17.44	6.900	3
18.85	PRO	(-)	(-)	(-)
23.58	GLY	23.64	2.105	1
24.99	ALA	25.06	4.720	2
28.18	CYS	(-)	(-)	(-)
29.32	VAL	(-)	(-)	(-)
31.50	MET	(-)	(-)	(-)
34.03	ILE	(-)	(-)	(-)
35.18	LEU	35.19	4.867	2
36.45	NLEU	36.43	10.000	(-)
39.00	TYR	(-)	(-)	(-)
40.39	PHE	(-)	(-)	(-)
44.45	HIS	(-)	(-)	(-)
47.96	LYS	(-)	(-)	(-)
49.99	NH ₃	(-)	(-)	(-)
52.06	ARG	52.02	2.671	1

6.4.3. Amino acid analysis

Ten nmoles of the peptide was added to an acid-washed test tube containing a solution of 0.7ml of concentrated HCl, 0.5ml of gelatine (0.4mg/ml, w/v) and 0.2ml of Norleucine (100nmoles). This was frozen to -88°C in a dry ice/ethanol mixture and then thawed. The tube was evacuated and sealed and the sample hydrolysed for forty-eight hours at 110°C . It was then dessicated and reconstituted in equal volumes of 0.1M sodium borate pH10.0 and 0.2M citrate pH2.2. The sample was finally filtered through a $0.22\mu\text{m}$ filter and loaded onto the amino acid analyser by Mr A Cronshaw of the Department of Biochemistry, Edinburgh University.

The sample analysis was compared to that of a mixture of standard amino acids which had been treated identically. From the retention times obtained for each standard amino acid and that of the internal Norleucine standard, it was possible to determine which amino acids were present within the peptide, and the ratios of each. No tryptophan was detected as this amino acid is hydrolysed under the conditions used for this method (Penko et al 1974).

Table 6.4.1. shows the retention time for each standard amino acid; the retention time for each amino acid within the peptide; the relative concentration of each peptide amino acid and the empirical ratio of the peptide amino acids.

The amino acids detected in the peptide were in the expected ratios for the sequence which was synthesised, (except for tryptophan which was not detected). In order to be certain that the correct sequence had been synthesised and purified, the peptide was sequenced.

6.4.4. Sequencing the synthetic peptide

Ten μ l of the peptide mixture (0.1mg/ml) in 10% ethanol (approximately 1nmol) was applied to an Applied Biosystems Ltd automatic gas phase micro-sequencer by Dr L Gilmore, of the Department of Biochemistry, Edinburgh University.

The method used is a variation of the Edman degradation method (Mendes and Lai 1975), whereby the amino acids are sequentially removed from the N-terminus of the peptide. The amino acids are reacted with phenylisothiocyanate to form the relatively unstable phenylthiocarbamyl derivative which isomerizes to give the phenylthiohydantoin (PTH) derivative of the amino acid. Each derivatised amino acid is individually analysed and the retention time obtained for each is compared to that of standard amino acids treated under identical conditions. The amount of each amino acid is measured (in pmols) at each liberation step, and from this the sequence of the peptide is determined. Table

Table 6.4.2. The results of sequencing the synthetic peptide corresponding to the red and green cone pigment proteins.

The retention time for each amino acid was compared to that of an internal standard DPT (data not shown). The concentration of other amino acids present was not significant (data not shown) and the expected sequence was obtained for the peptide.

See text for experimental detail.

Amino acid (1st-11th)	retention time (mins)	pmols	amino acid
1st	11.48	671.01	ALA
2nd	7.03	368.97	GLN
3rd	7.03	348.96	GLN
4th	22.30	217.54	TRP
5th	6.72	178.64	SER
6th	24.58	310.95	LEU
7th	7.03	210.10	GLN
8th	14.47	SIG	ARG
9th	24.58	218.65	LEU
10th	11.48	183.01	ALA
11th	8.02	42.41	GLY

SIG at a significant level, no numerical value obtained.

6.4.2. shows the retention times and the amount of the major amino acid liberated at each sequential step. No other amino acids were detected at a significant level. In conclusion, the expected sequence was indeed observed and there was little evidence of contaminating sequences. to a larger protein (Goding 1983 and Weiss *et al.* 1987).

It was necessary to conjugate the peptide to a protein which itself is not present in the bovine retina so that antibodies raised against the protein conjugated to the peptide would not be detected in the screening process and so would not be confused with any anti-peptide antibodies produced.

Another criterion for choosing a suitable protein to conjugate to the heptan is that it should contain the chemical structures necessary to allow conjugation to be carried out easily.

In vivo, the N-terminus of the cone pigment protein is at the luminal face of the cone photoreceptor, those amino acids towards the C-terminus of the protein being embedded in the membrane (Nathans *et al.* 1986). To mimic this, the peptide was conjugated via its free carboxyl terminus, leaving its amino terminus free in solution. The alcohol soluble compound N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EDC) was used to link the peptide and the protein molecules.

6.4.5. Haptenising the antigen.

To increase its antigenicity, it was decided to conjugate the undecapeptide to a larger protein. In many cases small proteins or peptides have in many cases produced an immune response in animals after being haptenised to a larger protein (Goding 1983 and Weiss et al 1987).

It was necessary to conjugate the peptide to a protein which itself is not present in the bovine retina so that antibodies raised against the protein conjugated to the peptide would not be detected in the screening process and so would not be confused with any anti-peptide antibodies produced.

Another criterion for choosing a suitable protein to conjugate to the hapten is that it should contain the chemical structures necessary to allow conjugation to be carried out easily.

in vivo, the N-terminus of the cone pigment protein is at the luminal face of the cone photoreceptor, those amino acids towards the C-terminus of the protein being embedded in the membrane (Nathans et al 1986). To mimic this, the peptide was conjugated via its free carboxyl terminus, leaving its amino terminus free in solution. The alcohol soluble compound N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was used to link the peptide and the protein molecules.

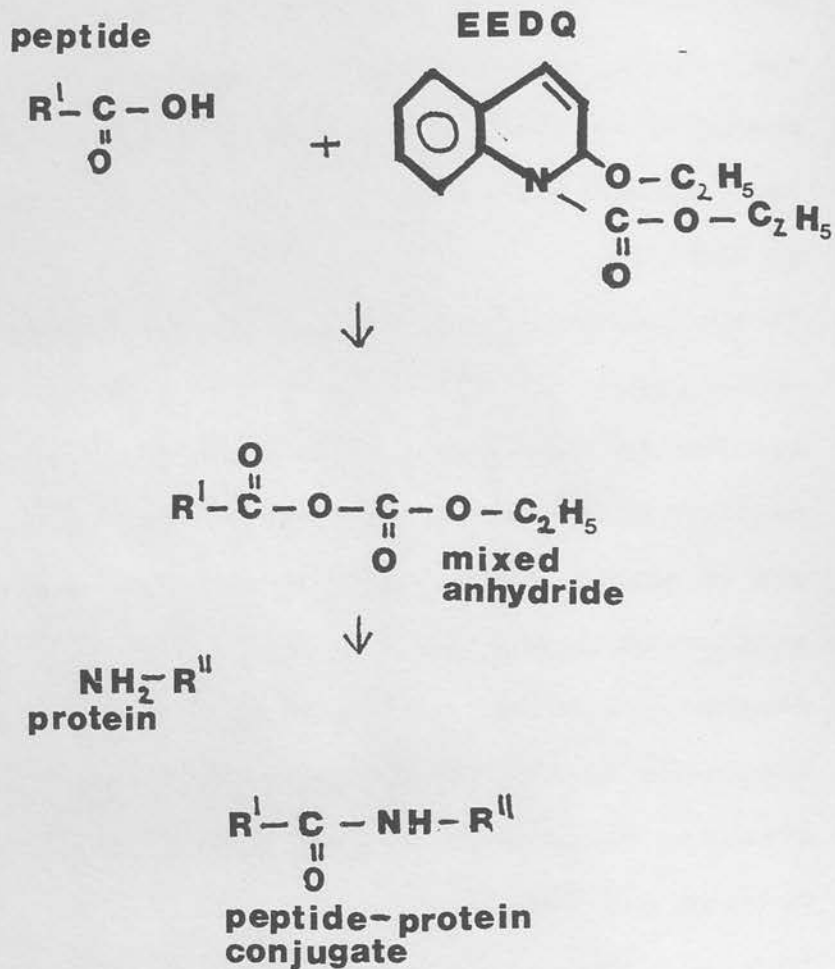


Figure 6.4.2. The reaction which takes place between EEDQ, the hapten and the protein to be conjugated. See text for experimental detail.

Two molecules were chosen for conjugating to the peptide (both absent from the bovine neural retina), egg albumin and polylysine (the latter containing a large number of free amino groups available for conjugation to the peptide).

EEDQ was added to a mixture of polylysine/ovalbumin and peptide with the latter in excess. In the polylysine/peptide mixture there were many more free amino groups on polylysine molecules than on peptide molecules (due to the large number of free amino groups on a single polylysine molecule, approximately 120). The addition of EEDQ to these should favour the coupling of the free carboxyl groups of the peptide molecules (present in excess of the free carboxyl groups of the polylysine molecules), to the free amino groups of the polylysine molecules (present in excess of the free amino groups of the peptide molecules) (Figure 6.4.2.). Some head-to-tail coupling would be inevitable and unavoidable but should have been minimised by the high ratio of peptide:polylysine molecules present. The EEDQ was added in excess to push the conjugation reaction towards completion. In ovalbumin, there are less free amino groups than in polylysine, so the likelihood of the desired conjugation taking place is less. It should be noted however that only a proportion of the reaction products need be the desired structure to be used for immunising mice. Monoclonal antibodies may be selected which show immunoreactivity against the cone

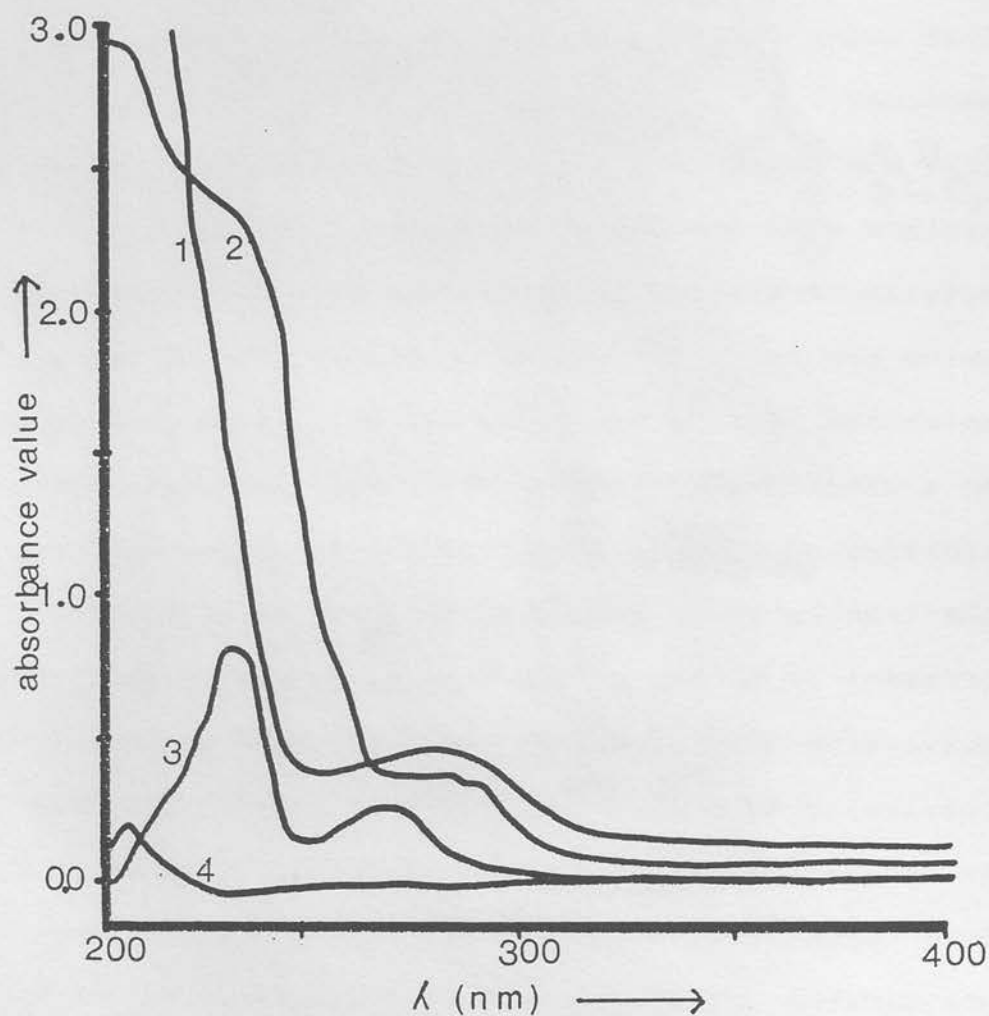


Figure 6.4.3 The absorbance profiles between 200nm and 400nm, of the peptide, EEDQ, polylysine and the peptide-polylysine conjugate.

1. peptide-polylysine conjugate (undiluted)
2. peptide (0.1mg/ml, 10% ethanol)
3. EEDQ (1mg/ml, 100% ethanol)
4. polylysine (1mg/ml, 25% ethanol)

photoreceptor cells in the retina, whereas those monoclonal antibodies raised against any other product of the conjugation procedure should not be detected in the screening process and so would be discarded.

0.4 moles of the peptide (in ethanol), was added to either 0.1 moles of ovalbumin or to 0.1 moles of polylysine (both in solution in distilled water). For the polylysine/peptide mixture, this gave an approximate ratio of free carboxyl groups on peptide:free amino groups on hapten molecules of 1:30. To each of these mixtures was added 1 mole of EEDQ (in ethanol) to give a final 50% ethanol solution. The mixtures were incubated overnight at room temperature and then dialysed against 50% ethanol to remove unreacted EEDQ and peptide from the mixture (material with MW under 12 KDalton).

The absorbance spectra (200-400nm) of the reactants and the products of the polylysine-peptide conjugation procedure were measured (Figure 6.4.3.). The polylysine and the EEDQ were found, as expected, to have little or no absorbance at 280nm and a higher absorbance towards the 200nm region, whereas the peptide had a higher absorbance at 280nm (due to the presence of tryptophan). The reaction products had a higher absorbance value at 280nm than did the reactants and this was taken to indicate the presence of peptide in the final mixture. This was assumed to have been conjugated to the polylysine since the unreacted peptide should have been removed in the dialysis.

These measurements were not carried out for the ovalbumin mixture since in this case both the hapten and the ovalbumin absorb light at 280nm. 2.4.5.1, and three cell fusions were carried out as described in Section 3.1.4. 2.4.5.2, 2.4.5.3, and 2.4.5.4. In the first fusion, the wells from 5.96-well plates were screened for anti-retinal antibodies using unfixed bovine neural retina sections. The ability of an anti-peptide antibody to bind to the native protein depends on the ability of the peptide to attain the conformation that the identical sequence assumes in the native protein (Wesshof *et al* 1984). To maximise the possibility of selecting clone-specific antibodies, changes in the structure of the proteins present in tissue sections were minimised by using unfixed tissue sections. Fixation of tissue may cause small changes in the structure of the proteins. Such changes may not affect the binding of antibodies with relatively high affinities to their antigen, but those with relatively low affinity may no longer bind the antigen if its structure is altered. This would be especially true for anti-peptide antibodies, which may have a high affinity for the synthetic antigen but would have a lower affinity for the native proteins. Four groups of 6 wells contained antibody with immunoreactivity against specific cell layers of the retina, three of these with the outer retina and one with the outer plexiform layer only and these were

6.4.6. Production of monoclonal antibodies

Mice were each immunised (3.1.1.) with a total of 30nmoles of each peptide conjugate (6.4.5.), and three cell fusions were carried out as described in Section 3.1.4.

In the first fusion, the wells from 5 96-well plates were screened for anti-retinal antibodies using unfixed bovine neural retina sections. The ability of an anti-peptide antibody to bind to the native protein depends on the ability of the peptide to attain the conformation that the identical sequence assumes in the native protein (Westhof et al 1984). To maximise the possibility of detecting cone-specific antibodies, changes in the structure of the proteins present in tissue sections were minimised by using unfixed tissue sections. Fixation of tissue may cause small changes in the structure of the proteins. Such changes may not affect the binding of antibodies with relatively high affinities to their antigen, but those with relatively low affinity may no longer bind the antigen if its structure is altered. This would be especially true for anti-peptide antibodies, which may have a high affinity for the synthetic antigen but would have a lower affinity for the native proteins.

Four groups of 6 wells contained antibody with immunoreactivity against specific cell layers of the retina, three of these with the outer retina and one with the outer plexiform layer only and these were

expanded into 24-well plates. A large number of the wells contained antibody with very general specificity which bound to all the cell layers of the retina. Four of these groups of 6 wells were also expanded into a 24-well plate. When screened individually, those wells previously containing antibody with immunoreactivity against specific layers of the retina, were found to no longer contain antibody secreting cells. One of the 24 wells containing the general anti-retinal antibody was further expanded and cloned. This was named B1 and was used in further experiments.

The second fusion was abandoned on Day 2 when it became obvious that the NSO cells had reverted to a HAT-insensitive state.

In the third fusion, the wells from six 96-well plates were screened and ten groups of 6 wells containing anti-retinal antibodies were expanded into 24-well plates. These were screened individually and 9 cell lines were further expanded. All of these cell lines secreted antibody with immunoreactivity against all the cell layers of the retina and no cone-specific antibodies were produced. For this reason these cell lines were not cloned and one was used in further experiments. This was named B2.

Table 6.4.3. The results of dot blotting experiments using different antigen solutions for the monoclonal antibodies B1, B2 or 1001.A3. See text for experimental detail.

<u>Antigen solution</u>	<u>Monoclonal antibody</u>			
	no Mab	B1	B2	1001.A3
IPM	(-)	(++)	(+++)	(+++)
TD	(+)	(+)	(++)	(+)
Oval	(++)	(++)	(++++)	(++)
Oval-pep	(-+)	(-)	(++)	(+)
P-lys-pep	(-)	(-)	(+)	(+)
IRBP	(-)	(-)	(+)	(-)
a1001.A3*	(-)	(-+)	(+)	(++)
PNA**	(-)	(-+)	(+)	(-)

TD = trypsin-digest

Oval = 1mg/ml ovalbumin

Oval-pep = peptide conjugated to ovalbumin

P-lys-pep = peptide conjugated to polylysine

* High molecular weight material isolated from IPM by gel filtration

**PNA-binding material isolated from IPM by gel filtration

(-) no binding

(-+) little binding

(+) binding (strength of binding denoted by the number of +'s)

6.4.7. Competitive binding studies

Tissue sections of the bovine neural retina were treated with the antibodies (2.2.1.) B1 and B2 except that the "anti-peptide antibodies" were preincubated and incubated in 100 μ l aliquots of ovalbumin or polylysine (1mg/ml)(w/v). This was done in an attempt to compete out antibody binding to the tissue sections with antigen in solution. Should the monoclonal antibodies bind to the ovalbumin or polylysine molecules, then the tissue sections should not bind to antibody and no fluorescence would be detected. Staining patterns and intensity of staining were identical to tissue sections stained to visualise anti-peptide antibody binding. This indicated that either the monoclonal antibodies were binding to antigenic sites in the retina which are not present on ovalbumin/polylysine molecules, or else they bind to the retina with a higher affinity than the antigenic sites present on these molecules.

6.4.7. Discussion

6.4.8. Immunodotting of anti-peptide antibodies

Immunodotting experiments were carried out (3.2.1.) using polylysine, ovalbumin or the peptide-protein conjugates as the antigen solutions (1mg/ml)(w/v). Control dots using antibody diluting solution (2.2.1.) to replace the B1 or B2 antibody culture media, showed non-specific binding occurring (Table 6.4.3.). This must be between the polylysine/ovalbumin and a component

of the incubation solutions. No conclusions can be drawn from these immunodotting experiments in which the B1 and B2 antibodies showed immunoreactivity with both the ovalbumin and the polylysine (Table 6.4.3.). This suggested the ovalbumin and the polylysine may bind non-specifically to component(s) present in the 2nd antibody solution or in the streptavidin-HRP conjugate solution. B1 and B2 both showed immunoreactivity with the IPM, which was not seen in the control dot experiments. B2 showed immunoreactivity with every antigen solution it was tested against, whereas B1 showed weak immunoreactivity against some of these (Table 6.4.3.). From the intensity of staining it seems the B2 culture medium has a much higher antibody titre than does B1, and binds with less specificity to a wider range of substances than B1. The non-specific binding makes it difficult to distinguish any specific immunoreactivity of the antibodies.

6.4.9. Discussion

The staining patterns of the antibodies B1 and B2 on tissue sections of the bovine neural retina indicate that the "anti-peptide" antibodies bind many components of cells within the retina, other than the cone photoreceptors. This is most probably due to non-specific binding of the antibodies to the tissue, rather than the presence of cone-like proteins throughout the retina.

It was assumed that the synthetic undecapeptide would adopt the same conformation in vitro as does the corresponding sequence of the native protein in vivo. This may not be the case and antibodies may have been produced with strong immunoreactivity against the synthetic peptide but with very weak immunoreactivity against the sequence in vivo. Such antibodies would not have been detected in the screening process used. It should be noted that the synthetic peptide corresponds to the N-terminus of a human protein and that the monoclonal antibodies produced were tested for their immunoreactivity against bovine retina. It was assumed that differences in the primary structure of the protein, between species would be minimal (as is the case between human and bovine rhodopsin) (Nathans et al 1986), and that an antibody with immunoreactivity against the protein in one species would cross-react with the protein of the other species. It proved impossible to obtain human retinas and so no human tissue sections could be treated with the "anti-peptide" antibodies. Differences in the structure of the protein between the species, may cause a reduction in binding of the monoclonal antibodies to the bovine tissue being tested.

SECTION 7: SUMMARY

Section 7 Final remarks and conclusions.

Three methods have been discussed by which attempts were made to produce monoclonal antibodies specific for molecules present on the surfaces of the rod and cone photoreceptor cells, or present in the IPM but associated with the photoreceptor cells. In the first method a crude antigen mixture was used to immunise mice. This method seemed least likely to succeed in producing the cell-specific monoclonal antibodies required, as it very much depended on luck as to whether the desired antibodies were produced. The second and third methods involved the purification of antigen, or the chemical synthesis of pure antigen for immunisation of mice. These approaches seemed more likely to be successful as the chances of producing less specific antibodies should have been reduced. In practice, however, the use of crude antigen mixtures resulted in the production of two interesting monoclonal antibodies whereas the use of the purer antigen mixtures and the synthetic antigen failed to result in the production of any interesting antibodies. The first method was the easiest to carry out in that no purification steps of the antigen were needed and the selection of photoreceptor and IPM-specific antibodies was carried out in the screening process. The initial selection process was based on treatment of tissue sections with the putative antibody-containing culture

supernatants. This allowed antibodies which bound specifically to the photo-receptors or to the IPM to be identified and the cells producing them to be expanded and cloned for further antigen analysis to be carried out. This seemed the most efficient screening process available. It made possible the screening of six hundred culture supernatants simultaneously. The use of tissue sections also rules out, or at least reduces, the possibility of false positives in that it would be unlikely that non-specific binding would be limited to a specific area within the tissue sections, whereas a false positive dot blot or Western blot is quite likely to occur and not be recognised as such immediately, leading to much unnecessary work and frustration. Some workers have used immune precipitation techniques in their initial screening. This would appear an excellent method (albeit tedious) and would select only high affinity antibodies. It does, however assume that the antigen is suitable for detecting by the methods to be used (usually gel electrophoresis and Western blotting) and that it is present as a single soluble species and not as an aggregate. In my case it is unlikely that such a screening method would have resulted in the detection of any useful antibodies as those antibodies which were detected by screening on sections appeared to have low affinity for the antigen or to bind to molecules not suitable for SDS-PAGE detection methods. This of course assumes that all the antibodies present

were in fact detected by the tissue section method (that is, not masked by aldehyde fixation) although one would expect high affinity monoclonal antibodies to be detected by all screening methods.

The general approach taken in this project to detect cell-specific molecules of the retina was carried out using bovine tissue, due to a readily available supply. There are questions to be asked about the direct relevance of work carried out using this tissue with respect to human tissue and human retinal abnormalities. The lack of availability of human tissue was a great drawback, as no direct comparisons could be made between the species. Such problems may be solved in the future by the use of immortal cell lines derived from human retinal cell types, such as photoreceptor cells. Such cell lines may provide a model system for the study of these cells without the need for biopsy or autopsy tissues.

This project was carried out with the aim of producing monoclonal antibodies against molecules on, or associated with, the surfaces of the photoreceptor cells with the view to characterising these molecules. Further studies would then be possible to consider the role these molecules play in the development of the adult tissue or in tissues affected by disease.

The locations and quantities of the molecules could be compared in normal adult tissue, developing tissue and diseased tissue in different species in which the antibodies cross-react. Monoclonal antibodies raised specifically against cone or rod cells can be used as markers to distinguish unequivocally between these cell types.

Two interesting monoclonal antibodies have been raised. The first, 1001.A1, binds the interstitial retinol binding protein (IRBP), the major glycoprotein of the interphotoreceptor matrix (IPM). This glycoprotein is synthesised by the rod photoreceptor cells and secreted into the IPM. In reality, many different monoclonal antibodies were raised against this glycoprotein and these may prove to be very useful tools for the identification of specific fragments within the polypeptide structure. Antigenic analysis has not yet been carried out. Digestion of the purified IRBP molecules with cyanogen bromide or proteolytic enzymes followed by characterisation of the antibody-binding specificities with respect to the purified peptide fragments would be possible, for example, using competitive ELISA techniques. This knowledge may be put to use in studies whereby the effects of smaller, defined fragments of the complete molecule are being tested, for instance in their ability to induce experimental uveitis. Here polyclonal antibodies could

not be used as they will not differentiate between the isolated fragments. However, should the antibodies all be specific for a particular fragment of the full length polypeptide, this may have important implications with respect to immunological studies of the retina and RPE with respect to this molecule. the distribution of the 1001.A3 antigen with respect to the rod and cone

The second monoclonal antibody, 1001.A3, which was raised against a soluble chondroitin sulphate proteoglycan within the IPM proved to be very interesting. Immunohistochemistry revealed the antigen to be present around the inner and outer segments of the photoreceptor cells. It is not clear from the antigen as histochemical data obtained whether the antigen is specific for the rod photoreceptors or whether it is associated also with the cone photoreceptor cells. The antigen is mostly soluble and much of it is removed by washing the retina. The insoluble fraction remains associated with the photoreceptors but not specifically with the cones. Washed bovine retina treated with urea of antibodies raised against chondroitin-6-sulphate have molecules shows a similar binding pattern. There is also a soluble fraction of chondroitin-6-sulphate molecules, present in IPM. In primates, chondroitin-6-sulphate molecules are associated specifically with the cone photoreceptors, whereas in rodents, chondroitin-6-sulphate molecules are associated with the photo-receptors in a more general manner, not specifically

with the cones. It may be that in primates, the 1001.A3 antigen is associated only with the cone photoreceptors whereas in non-primates, the 1001.A3 antigen is more generally distributed, as are chondroitin-6-sulphate-containing molecules. Double labelling experiments using PNA and 1001.A3 may show the distribution of the 1001.A3 antigen with respect to the rod and cone photoreceptor cells in both washed and control retina.

There is a very great deal of work still to be done making use of the 1001.A3 monoclonal antibody. Only the initial characterisation of the antigen has been carried out. One problem associated with the 1001.A3 antigen is that it does not appear to stain with the protein and carbohydrate-specific stains used to date and has only been identified by the ability of 1001.A3 to bind to it. Enzymatic treatment which degrades the structure of the antigen tends to abolish any immunoreactivity and therefore the molecule is no longer detectable. This has hindered progress in characterising the structure of the molecule more fully. It would be helpful to have available monoclonal antibodies which recognise different structures within the antigen. Antibodies specific for the core protein of the molecule for example, would be very useful to allow the polypeptide structure to be characterised. Future work should involve the production of monoclonal antibodies against the partially purified 1001.A3 antigen, which has been

subjected to treatment with chondroitinase ABC or hyaluronidase, for example. The availability of antibodies against both the complete structure and against the enzymatically-treated molecules would be very useful and should be relatively easily achieved. If it is possible to identify the core protein by means of a second antibody, and to remove the glycosaminoglycan side chains of the proteoglycan from the polypeptide, the purification of the core protein should be possible. This should be done with the view to obtaining at least a partial sequence of the structure. The knowledge of a partial amino acid sequence, together with monoclonal antibodies raised against the core protein, and also the complete structure of the proteoglycan molecule would be a very powerful combination. For example, cDNA expression libraries of the neural retina or of the retinal pigmented epithelium (RPE) may be screened with the antibodies in order to isolate the gene for this molecule. Antibodies raised against the core protein would be much more likely to succeed in this detection process due to the fact that 1001.A3 recognises post-translational modifications of the molecule which may not be produced, or may not be produced in the same way, in the expression vectors. However, without the knowledge of where the 1001.A3 antigen is synthesised one cannot be sure of which cDNA library to use.

Experiments involving the culture of pigmented epithelium cells or retinal cells, followed by tests to establish whether the 1001.A3 antigen is secreted by any of these cell types, may establish the source of the antigen. A partial amino acid sequence of the core protein may provide another method to find the cell types involved in synthesising the antigen. Various approaches are available, such as the construction of a collection of oligonucleotide probes for use in in situ hybridisation studies. This would allow studies to be carried out on the messenger RNA molecules of the core protein and hence the site of synthesis may be found. This would verify the tissue of origin of the core protein and would indicate which expression libraries would be suitable for use. These expression libraries could also be screened using the oligonucleotides in favour of the antibodies. Isolation of the gene would of course open up a whole new field of study, enabling gene sequencing and chromosome mapping to be carried out. Comparisons may be made with other core protein structures, possibly leading to clues as to the function of the molecule. Chromosomal location would make it possible to determine whether the proteoglycan core protein is linked to known human disease loci, for example retinitis pigmentosa, in which diseases have been linked to chromosomal locations but where the actual gene involved is unknown.

The 1001.A3 monoclonal antibody should be tested for immunoreactivity with other species. It is known to bind specifically to rat tissue, and other species such as human and mouse should also be tested. The ability to use rodent tissue would open up new investigative possibilities. It would be possible to carry out a developmental study of the mouse retina, during gestation and also during the period after birth in which the retina continues to develop. Animal models of human disease may also be used, such as the rd mouse and the RCS rat. This may prove interesting as particularly abnormalities of the composition of the IPM have been implicated in such diseases. Changes in the structure or quantity of the 1001.A3 antigen could be monitored and compared to the normal developmental pattern.

retina by treatment with trypsin. Previously it had

Finally it may be possible to continue the studies of the 1001.A3 antigen by the more direct method of immunohistochemistry using electron microscopy. This is a very specialised technique involving fixation, embedding and sectioning expertise. It may be possible to study the structure of the 1001.A3 antigen much more closely using the high resolution of electron microscopy. Conditions may be found to enable this molecule to be studied without loss of immunoreactivity. This may lead to the verification of the exact location of the antigen with respect to the rod and cone photoreceptors, as it would be possible to distinguish

the rod and cone cells and to find whether binding is common to both or restricted to a single type of photoreceptor. This method may also elucidate where the proteoglycan is synthesised, whether this occurs within the photoreceptor cells or within the RPE.

Several attempts were made to raise monoclonal antibodies against the PNA-binding glycoproteins associated with the cone photoreceptor cells. No such antibodies were raised which led to the conclusion that it is possible that these molecules are not particularly antigenic and that perhaps the production of antibodies against these molecules is not the best approach to take towards characterising them. It was found that these PNA-binding glycoproteins may be released from the retina by treatment with trypsin. Previously it had proved difficult to even partially purify these molecules from the retina since they are so tightly attached, as opposed to the soluble components of the IPM which were easily isolated. Further studies however indicated that the soluble IPM does in fact contain small quantities of these molecules, normally found associated with the cone photoreceptor cells. These are present in the IPM in the form of PNA-binding glycoproteins and chondroitin-6-sulphate containing molecules. A sensible approach would therefore be to purify these molecules from the IPM or the trypsin digest, using conventional chromatography methods. The

use of conventional chromatography would be especially suited to the isolation of the PNA-binding glycoproteins. The use of a narrower range gel filtration columns and/or combinations of ion exchange, reverse phase, or hydrophobic interaction chromatography may lead to the purification of these molecules to the point whereby it is possible to obtain a partial amino acid sequence of the glycoproteins. From this it may be possible to take the approach of obtaining oligonucleotide probes in order to carry out in situ hybridisation studies and studies at the gene level, as discussed above, with respect to the core protein of the 1001.A3 antigen. Another approach would be to synthesise synthetic peptides corresponding to sequences within the polypeptide backbones of the glycoproteins, in order to raise monoclonal or polyclonal antibodies against the complete protein. To increase the chances of success, it would be important to obtain synthetic sequences of 20-30 amino acids to ensure that any antibodies produced against the synthetic peptide, also bind to the full length protein with some degree of avidity. Such antibodies would allow studies to be carried out on the PNA-binding glycoproteins, independently of their glycosylation state. It is possible that these molecules are also present in non-glycosylated forms or with varying degrees of glycosylation, and such states would not be distinguished by the PNA lectin. Antibodies would also

distinguish between the different PNA-binding glycoproteins whose localisation and concentrations may be very different within the tissue. PNA would also not distinguish between these.

The final approach taken to raise monoclonal antibodies, specific for the photoreceptors was to use a synthetic antigen in the form of a synthetic peptide corresponding to the amino acid sequence of the N-terminus of cone-pigment proteins. No cone-specific monoclonal antibodies were produced by this method, probably due to the short length of the peptide antigen. Antibodies may have been produced with high affinities to the synthetic antigen, but which were not detected in the screening process due to their low affinities to the full length protein in vivo . If such an approach was to be taken again, a longer synthetic antigen should be used. This may lead to the production of antibodies with high affinities to both the synthetic antigen and to the full length protein. This has already been proved possible with the cone pigment proteins (Lerea et al 1989).

An alternative approach to raising antibodies against proteins such as the cone pigment proteins, of which the gene sequence is known, is to produce a synthetic fusion protein in bacteria to use as the antigen. This method has the advantage that large quantities of the whole protein sequence are used, leading to the production of high affinity antibodies to the protein in vivo. A

fusion protein may be constructed either by using a genomic clone of the gene, or if this is unavailable, by using the polymerase chain reaction (PCR) to amplify the gene sequence required. RNA (isolated from tissue which expresses the gene product) may be used as the starting material, to produce a small quantity of cDNA by means of the reverse transcriptase enzyme. PCR may then be used to produce a large quantity of the gene sequence required which can be put directly into an expression vector. If a promoter such as that of the β -galactosidase gene or the protein A gene is used, expression of the fusion protein in *E. coli* cells may be maximised by induction of the promoter. Fusion proteins have been made successfully for example, of the inhibitory sub-unit of rod cGMP phosphodiesterase. In this case large amounts of the protein, corresponding to that of approximately 2500 bovine eyes were purified (Brown and Stryer 1989).

In conclusion, as discussed above, the production of cell-specific monoclonal antibodies may provide very useful tools which enable the distribution of a particular molecule to be studied. Such antibodies may also be used to screen cDNA expression libraries. This may determine which cDNA clones are cell-specific where the antigen may not be known, or may determine those specific for a known protein, for example IRBP (Barrett

et al 1985). Gene analysis of the antigen is then possible. In this way molecular biology techniques and the use of monoclonal antibodies may be complementary.

Abbreviations

acetic acid:	glacial acetic acid
BSA:	bovine serum albumin
cDNA:	cloned deoxyribonucleic acid
cGMP:	cyclic guanosine monophosphate
CO ₂ :	carbon dioxide
gGDP:	deoxyguanosine diphosphate
gGTP:	deoxyguanosine triphosphate
dTDP:	deoxythiaseine diphosphate
dTMP:	deoxythiaseine monophosphate
dTTP:	deoxythiaseine triphosphate
DNA:	deoxyribonucleic acid
EDTA:	ethylenediamine tetra acetic acid
FITC:	fluorescein isothiocyanate
GDP:	guanosine diphosphate
GTP:	guanosine 3'-triphosphate
HCl:	hydrochloric acid
HEPES:	N-(2-hydroxyethyl)piperazine-N'-2-ethane sulphonic acid
H ₂ O ₂ :	hydrogen peroxide
HPLC:	high performance liquid chromatography
KDa:	kilo daltons
M:	Molar
MAb:	monoclonal antibody
MESE:	monophosphoric sulphonic acid
mg:	milligramme
mM:	millimolar
M _r :	molecular weight
nmol:	nanomole
nm:	nanometre
OS:	outer segments
pI:	isoelectric point
pmol:	pico mole
PMSE:	phenylmethylsulphonyl fluoride
R _i :	relative mobility with respect to the tracker dye
rRNA:	ribonucleic acid
RPE:	retinal pigmented epithelium
SDS:	sodium dodecyl sulphate
TBS:	tris buffered saline
TWEEN 20:	polyoxyethylenesorbitan monolaurate
U:	international enzyme unit
μCi:	micro curie
μg:	microgramme
μl:	microlitre
μM:	micromolar
γglobulin:	immunoglobulin

It should be noted that throughout the text the Greek character μ is denoted u.

Abbreviations

acetic acid:	glacial acetic acid
BSA:	bovine serum albumin
cDNA:	cloned deoxyribonucleic acid
cGMP:	cyclic guanosine monophosphate
CO ₂ :	carbon dioxide
dGDP:	deoxyguanosine diphosphate
dGTP:	deoxyguanosine triphosphate
dTDP:	deoxythiamine diphosphate
dTMP:	deoxythiamine monophosphate
dTTP:	deoxythiamine monophosphate
DNA:	deoxyribonucleic acid
EDTA:	ethylenediamine tetra acetic acid
FITC:	fluorocein isothiocyanate
GDP:	guanosine diphosphate
GTP:	guanosine 5'-triphosphate
HCl:	hydrochloric acid
HEPES:	N-(2-hydroxyethyl)piperazine-N'-2-ethane sulphonic acid
H ₂ O ₂ :	hydrogen peroxide
HPLC:	high performance liquid chromatography
KDa:	Kilo daltons
M:	Molar
Mab:	monoclonal antibody
MES:	morpholino-ethane-sulphonic acid
mg:	milligramme
mM:	millimolar
Mr:	molecular weight
NH ₃ :	ammonia
nm:	nanometre
OS	outer segments
pI:	isoelectric point
pMols:	pico moles
PMSF:	phenylmethylsulphonyl fluoride
R _r :	relative mobility with respect to the tracker dye
RNA:	ribonucleic acid
RPE	retinal pigmented epithelium
SDS:	sodium dodecyl sulphate
TBS:	tris buffered saline
TWEEN 20:	polyoxyethylenesorbitan monolaurate
U:	international enzyme unit
uCi:	micro curie
ug:	microgramme
ul:	microlitre
uM:	micromolar
γglobulin:	immunoglobulin

It should be noted that throughout the text the Greek character μ is denoted u.

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